University of Alberta

Multiple intracellular calcium stores regulate physiological functions in neuroendocrine cells

by

James Daniel Johnson ©

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in

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c/o Ken and Sally Johnson
178 Warren Road, RR 4
Lanark, Ontario, Canada, K0G 1K0

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Multiple intracellular calcium stores regulate physiological functions in neuroendocrine cells submitted by James Daniel Johnson in partial fulfillment of the degree of Doctor of Philosophy in Physiology and Cell Biology.

J.P. Chang, Supervisor

J.I. Golberg, Committee member

G.G. Goss, Committee member

F.W. Tse, External examiner

J.K. Foskett, External examiner

S.D. Campbell, Chairperson and examiner

September 27, 2000

Abstract

Ca^{2+} signals are used by all cells to transmit information and are known to control many cellular functions. How Ca^{2+} signals selectively regulate specific cellular functions is not well understood. In theory, specificity may be imparted by spatio-temporal Ca^{2+} signal coding and the involvement of multiple Ca^{2+} stores. However, whether agonist- and function-specific intracellular Ca^{2+} stores control hormone release and production has not been systematically studied in neuroendocrine systems. Single-cell Ca^{2+} imaging, acute measurements of hormone release, long-term measurements of hormone production, storage and secretion, as well as mRNA levels, were used to test the hypothesis that multiple intracellular Ca^{2+} stores are physiologically important in gonadotrope and somatotrope cells of the goldfish pituitary. These Ca^{2+}-dependent cellular functions were examined in the presence of endogenous regulators drugs known to modulate a variety of Ca^{2+} stores.

It was found that both cell types are capable of generating agonist-specific Ca^{2+} signals. The rate of rise, but not amplitude, of the Ca^{2+} signals appeared to encode information important for the control of hormone secretion. In the case of the two endogenous gonadotropin-releasing hormones, differential control of Ca^{2+} signalling and cell function, may result from the activation of different sets of Ca^{2+} stores. Several Ca^{2+} signalling systems controlling hormone secretion, including those sensitive to ryanodine or caffeine, can be altered with respect to the reproductive status of the animals. Also, a role for mitochondrial Ca^{2+} uptake in the control of basal Ca^{2+} homeostasis and hormone release was described. Differential involvement of pharmacologically distinct Ca^{2+} stores in basal and agonist-stimulated hormone release suggests the presence of at least five functionally-independent Ca^{2+} stores in both cell types. Moreover, by comparing the roles of two independent Ca^{2+} stores in cellular functions involved in the production and secretion of hormones, I provide the first evidence that function specificity in

neuroendocrine cells may be imparted through the involvement of multiple intracellular Ca^{2+} stores.

This is the first study of its kind to examine the role of multiple Ca^{2+} stores in processes involved in basal and agonist-stimulated hormone release in any cell type.

"The purpose of it's not unique
not to build masterpieces
for a delectative elite
but simply to wake to your life...

...and there's a sign of life in this play
not to get order from chaos
or tell you how to create
but simply to wake to your life"

Adapted from "Tiger The Lion" by the Tragically Hip, (Universal Music 2000), which contains text adapted from; *Off The Wall – RobertRauschenberg and the Art World of our Time* by Calvin Tomkins, (Doubleday and Company, Inc., 1980) and elements paraphrased from a John Cage lecture; Silence: Lectures and Writings (Wesleyan University Press, Middletown, Conn., 1961.

Preface

The primary goal of this thesis will be to use concepts derived from state-of-the-art cell biology and biophysics literature to answer questions about the physiology of Ca^{2+} signalling in a model neuroendocrine system. Thus, this thesis contains two inter-related and equally important components. The first part of this volume (Chapters 1) contains a comprehensive, current and critical assessment of the potential complexity of Ca^{2+}-dependent signal transduction in neuroendocrine systems. From this information, several novel hypotheses can be put forward, including the possibility that agonist- and function-specific Ca^{2+} stores may control multiple aspects of the extended secretory pathway.

Chapter 2 is a specific introduction, where the known signal transduction cascades mediating agonist-stimulated hormone release in my chosen experimental models, goldfish gonadotropes and somatotrope, are highlighted and reviewed. These cell types were chosen because Ca^{2+} signals and Ca^{2+}-dependent cellular functions can be readily measured and because both cell types share two neuropeptides (gonadotropin-releasing hormones; GnRH) as common neuroendocrine regulators. The latter feature allows for easy comparisons of agonist-specific signalling between and within cell types. I have framed this specific introduction in the context of other model systems, specifically mammalian pituitary cells.

After a description of the methods used herein (Chapter 3), Chapters 4-12 provide complete accounts of specific projects. These include studies on: 1) agonist-specific Ca^{2+} signalling in gonadotropes, 2) the roles of agonist-specific Ca^{2+} stores in GnRH-stimulated hormone release in both cell types, 3) the involvement of ryanodine-sensitive Ca^{2+} stores in both cell types, 4) the involvement of mitochondrial Ca^{2+} buffering in the control of hormone release, 5) the importance of the rate of rise of Ca^{2+} signals in the control of gonadotropin release, and 6) the role of multiple function-specific Ca^{2+} stores in the control of several Ca^{2+}-dependent events along the extended secretory pathway. Each data chapter has its own discussion section which puts the results in context of other research in the field and discusses the physiological relevance of the specific findings. A broader discussion of the significance of the entire thesis, as well as updated working models of the signal transduction in goldfish gonadotropes and somatotropes is found in Chapter 13.

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I also gratefully acknowledge the participation of my colleagues in acquiring or analyzing some data in this thesis. Specifically, data shown in Figure 4.2 was obtained by recalculating raw, unpublished data recorded by Dr. Fred Van Goor. Special thanks to Christian Klausen in Dr. Hamid Habibi's laboratory (University of Calgary) for teaching me the Northern Blot technique and for doing 2 of the thapsigargin replicates in Figures 11.6 and 12.6. The vector method of quantifying spatial features of Ca^{2+} signal was developed in collaboration with Dr. Calvin Wong. This approach would have remained in our imaginations without his mathematical prowess.

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I am a lucky man. I have the most loving and supportive family and the coolest group of friends. My parents raised me to believe that there are no limits with regard to what I could do. This gift of confidence is a special gift indeed. I was also lucky enough to grow up with a brilliant thinker in my brother, Chuck.

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Effect of VGCC activation on GH mRNA levels

13.1 Agonist- and function-specific Ca^{2+} signalling in gonadotropes

Agonist- and function-specific Ca^{2+} signalling in somatotropes

Abbreviations

BAPTA-(AM)	2-bis-(2-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (tetraacetoxymethyl ester)
BHQ	2,5-di-(t-butyl)-1,4-hydroquinone
$[Ca^{2+}]_C$	cytosolic free calcium concentration
$[Ca^{2+}]_M$	mitochondrial free calcium concentration
$[Ca^{2+}]_L$	lumenal free calcium concentration
$[Ca^{2+}]_{SG}$	secretory granule calcium concentration
cADPR	cyclic adenosine diphosphate ribose
CaM	calmodulin
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cfGnRH	catfish gonadotropin-releasing hormone
cGnRH-II	chicken gonadotropin-releasing hormone-II
CICR	Ca^{2+}-induced Ca^{2+} release
CPA	cyclopiazonic acid
DAG	1,2-diacylglycerol
DMSO	dimethyl sulphoxide
ER	endoplasmic reticulum
G-protein	guanine nucleotide-binding protein
GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRP-6	growth hormone-releasing hexapeptide
GTH	gonadotropin (luteinizing hormone and follicle stimulating hormone)
GTH-II	gonadotropin II
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
NAADP	nicotinic acid-adenine dinucleotide phosphate
NAADPR	nicotinic acid-adenine dinucleotide phosphate receptor
NCX	sodium-calcium exchange
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
MGnRH	mammalian gonadotropin-releasing hormone
PACAP	pituitary adenylate cyclase-activating polypeptide
PMCA	plasma membrane Ca^{2+}-ATPase
PLC	phospholipase C
PLA_2	phospholipase A_2
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
RR	ruthenium red
Ry	ryanodine
RyR	ryanodine receptor
S1P	sphingosine 1-phosphate
SCaMPER	sphingolipid Ca^{2+} release-mediating protein of endoplasmic reticulum
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
sGnRH	salmon gonadotropin-releasing hormone
SOC	store-operated Ca^{2+} channels
SPC	sphingosylphosphorylcholine
SL	sphingolipid
Tg	thapsigargin
TMB-8	8-(N,N-diethyl-amino)octyl 3,4,5-trimethoxybenzoate hydrochloride
TPA	12-O-tetradecanoylphorbol 13-acetate
TRP	transient receptor potential
VGCC	voltage-gated Ca^{2+} channels

Chapter 1.

Function- and agonist-specific Ca^{2+} signalling: the requirement for and mechanism of spatial and temporal complexity in Ca^{2+} signals*

Ca^{2+} signalling is a ubiquitous, multifunctional and conserved mechanism for the transduction of information in living cells (Clapham, 1995; Petersen et al., 1994; Berridge, 1997; Trewavas et al., 1996). Ca^{2+} signals can be defined as controlled deviations from the resting cytosolic Ca^{2+} ion concentration ($[Ca^{2+}]_c$). Since prolonged elevations in $[Ca^{2+}]_c$ are lethal (Clapham, 1995; Carafoli, 1987; Choi, 1992), cells expend considerable energy maintaining very low cytosolic Ca^{2+} levels (typically 50-200 nM free Ca^{2+}) compared to the extracellular environment which is in the millimolar range (Clapham, 1995; Carafoli, 1987). This steep gradient, accomplished by extrusion of Ca^{2+} from the cytosol into the extracellular space and intracellular organelles, combined with the presence of Ca^{2+}-selective ion channels on the plasma and organelle membranes, allows Ca^{2+} signals to be generated rapidly.

The purpose of this chapter is not to comprehensively review the entire literature of Ca^{2+} signalling. Instead, I shall endeavor to focus on recent biophysical, biochemical and theoretical advances in the study of signal transduction involving Ca^{2+} signals. A limited number of examples, primarily from the biochemical and biophysical literature, will be used to illustrate the potential complexity of Ca^{2+} signalling. Another goal of this chapter is to provide insight as to how these fundamental data on the complexity of Ca^{2+} signalling can be applied to the study of neuroendocrine regulation. Unfortunately, studies that incorporate an appreciation for multiple Ca^{2+} stores and the diversity of Ca^{2+}-dependent target functions are rare at the physiological level. In many cases, only speculation on the physiological relevance is possible at this time.

This review chapter is organized into three major sections. The first section will outline the problem. How does such a ubiquitous signalling molecule as Ca^{2+} ion relay information, with specificity, to a great variety of cellular functions? Second, I will examine the ultrastructural and molecular heterogeneity of Ca^{2+} sources, with special emphasis on multiple novel intracellular Ca^{2+} stores. Third, I will examine whether cells generate Ca^{2+} signals with sufficient complexity to control multiple cellular functions by looking at examples of spatial and temporal Ca^{2+} signal coding.

Section I: Multiple Ca^{2+}-Dependent Signalling Cascades and Cellular Functions

Diversity of Ca^{2+}-dependent cell functions

Individual cells activate multiple Ca^{2+}-modulated outcomes through the selective regulation of a bewildering number of Ca^{2+}-regulated enzymes. The

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* A version of this chapter can be found in, Johnson JD, Chang JP. 2000a. Function- and agonist-specific Ca^{2+} signalling: the requirement for and mechanism of spatial and temporal complexity in Ca^{2+} signals. Biochem. Cell Biol. 78, 217-240.

spectrum of Ca^{2+} involvement ranges from permissive to triggering actions. One ubiquitous and conserved enzyme that has received particular attention is calmodulin (CaM), which binds Ca^{2+} through conserved motifs known as EF hands (Weinstein and Mehler, 1994). CaM is probably the major physiologically active Ca^{2+} binding protein in most cells (Braun and Schulman, 1995). Recent evidence suggests that CaM can faithfully transduce the spatial and temporal complexity of Ca^{2+} signals (Inagaki et al., 1997; Craske et al., 1999). CaM can regulate the activity of many of enzymes. Among the targets for CaM are components of other signal transduction cascades (Braun and Schulman, 1995).

Many researchers have focused on pathways regulated by Ca^{2+}-dependent kinases, such as CaMkinases (CaMK), and phosphatases, such as calcineurin (Braun and Schulman, 1995; Yakel, 1997; Westphal et al., 1998). Once CaMK is activated, it prolongs the transduction of the information encoded by the Ca^{2+} signal, even after $[Ca^{2+}]_c$ has returned to basal levels (MacNicol et al., 1990). Ca^{2+}-dependent effects on cellular enzymes can be mediated either through direct binding of Ca^{2+} or through several classes of Ca^{2+} effector proteins, many of which contain either EF-hand motifs, as in CaM, or C2 domains such as those found in protein kinase C (PKC). Several key steps in other signal transduction pathways can also be modulated by Ca^{2+}, including: plasma membrane ion channels and transporters (Levitan, 1999, Tsien and Tsien, 1990), conventional PKCs (α, βI, βII, and γ isoforms, Newton, 1997), adenylate cyclases (Mons et al., 1998), phospholipase A_2 (PLA_2; Kramer and Sharp, 1997), phospholipase C (PLC; Singer et al., 1997), phosphodiesterases (Braun and Schulman, 1995), nitric oxide synthase (Griffith and Stuehr, 1995), mitogen-activated protein kinase cascades (Egea et al., 1999) and the protease, calpain (Saido et al., 1994). The physiological roles of several other classes of Ca^{2+}-binding proteins, including the S100 family (Schäfer and Heizmann, 1996) and the annexin family (Burgoyne and Geisow, 1989) are less well understood.

Although the list of signal transduction components presented above contains only a limited number of examples, it indicates clearly that Ca^{2+}-regulated signal transduction cascades may exert indirect, as well as direct, control over many cellular functions. The Ca^{2+} signalling cascades that participate in excitation-secretion coupling and excitation-contraction coupling are perhaps the most extensively studied (Neher, 1998; Kasai, 1999; Somlyo and Somlyo, 1994; Braun and Schulman, 1995). Ca^{2+} also plays important roles throughout the lifetime of cells/organisms, from fertilization (Gilkey et al., 1978) to death, whether it occurs through apoptosis, necrosis or excitotoxicity (Trump and Berezesky, 1996; Marks, 1997; Kruman and Mattson, 1999; Choi, 1992). A role for Ca^{2+} signalling has been suggested in various parts of the cell cycle (Hepler, 1994; Groigno and Whitaker, 1998) and in differentiation (Gu and Spitzer, 1995). Ca^{2+} has also been shown to be involved in cell adhesion and motility, cytoskeletal reorganization (Takeichi, 1990; Witman, 1993; Maxfield, 1993; Pettit and Fay, 1998; Janmey, 1994) and restructuring/repositioning of organelles, including Ca^{2+} stores such as the endoplasmic reticulum (ER; Subramanian and Meyer, 1997). Ca^{2+} is also an important intercellular signal, mediating cell-to-cell signalling through the actions of gap junctions (Sanderson, 1996) or Ca^{2+}-sensing

receptors, which are widely distributed (Shorte and Schofield, 1996; Nemeth, 1995; Chattopadhyay et al., 1996).

Cellular metabolism may also be linked to the regulation of Ca^{2+}-sensitive mitochondrial dehydrogenases (Hajnóczky et al., 1995), and possibly ATP synthase (Hubbard and McHugh, 1996). Hormonal stimulation of hepatocytes with vasopressin evokes cytosolic Ca^{2+} signals that in turn regulate mitochondria Ca^{2+} concentration ($[Ca^{2+}]_m$). Temporally resolved measurements of $[Ca^{2+}]_c$, $[Ca^{2+}]_m$ and the redox state of pyridine nucleotides have demonstrated that cellular metabolism is regulated by the frequency of Ca^{2+} oscillations (Hajnóczky et al., 1995; Robb-Gaspers et al., 1998). Similar techniques were used to demonstrate that metabolism in pancreatic β-cells is also controlled by Ca^{2+} oscillations. The regulation of cellular metabolic rate by Ca^{2+} signals is probably a general mechanism of ensuring that energy metabolism is up-regulated in activated cells (Pralong et al., 1994; Duchen, 1999).

In some cases, Ca^{2+} participates in opposite elements of the same physiological function. For example, in addition to the well known role of Ca^{2+} in triggering contraction, high-amplitude localized Ca^{2+} release events stimulate Ca^{2+}-activated potassium channels leading to relaxation in smooth muscle (Nelson et al., 1995). In the brain, Ca^{2+} plays an important role in both long-term potentiation and long-term depression of synaptic transmission (Svoboda and Mainen, 1999). Ca^{2+} affects several components of the secretory pathway, including both exocytosis and endocytosis (Klingauf et al., 1998; De Camilli and Takei, 1996; Lai et al., 1999). Many other parts of the secretory pathways can also be controlled independently, and thus require specific Ca^{2+} signals. This chapter will devote considerable attention to this issue, since it is of particular interest to neuroendocrinologists.

Ca^{2+} regulation of multiple sites on the protein synthesis/secretory pathway

Ca^{2+} is known to play a role in several elements of the extended secretory pathway, which includes hormone gene expression and synthesis. Many aspects of this pathway can be modulated independently, often as a consequence of hormonal control. Ca^{2+}-dependent gene expression can be regulated by a great number of positive and negative regulators of transcription, including the cyclicAMP response element-binding protein (CREB), serum response factor, c-Jun and many others (Shaywitz and Greenberg, 1999; Cruzalegui et al., 1999; Johnson et al., 1997; Roche and Prentki, 1994). These transcription factors integrate other signal transduction cascades with Ca^{2+}-dependent signalling (Shaywitz and Greenberg, 1999). The ubiquitous and pleiotropic transcription factor nuclear factor-κB (NF-κB; F. Chen et al., 1999) is a component of one of the best understood Ca^{2+} signalling pathways. As with NF-κB, studies on nuclear factor of activated T cells (NF-AT), which is also regulated by calcineurin, have provided insight into the regulation of multiple targets by Ca^{2+} signals (Crabtree, 1999). Stimulatory transcription factors are thought to be regulated indirectly by Ca^{2+}-activated kinase pathways (Bading et al., 1993). In contrast, Ca^{2+} binds directly to EF-hand motifs on the transcriptional repressor known as the downstream regulatory element antagonist modulator (DREAM) and releases it

from the downstream regulatory element, thereby derepressing the target gene (Carrion et al., 1999). Curiously, Ca^{2+} may modulate the transcriptional activity of members of the nuclear hormone receptor superfamily through calreticulin, a Ca^{2+}-binding lectin chaperone located in the ER (Burns et al., 1994; Perrone et al., 1999; Krause and Michalak, 1997). Taken together, these reports demonstrate that Ca^{2+} can act through many mechanisms to control gene expression. Whether these activities are widespread in neuroendocrine systems remains to be tested.

Ca^{2+} has been implicated in the control of protein synthesis. For example, CaMK III regulates protein synthesis via phosphorylation of elongation factor-2 (Braun and Schulman, 1995 and references therein). Ca^{2+} may also play a role in directly modulating the rate of initiation of protein translation (Brostrom and Brostom, 1998). Specifically, depletion of Ca^{2+} from the ER, and not the elevation of $[Ca^{2+}]_c$, inhibits the rate of protein synthesis (Kimball and Jefferson, 1992). In a variety of cell types, pharmacological or hormonal depletion of Ca^{2+} stores impedes protein processing in the ER. This event initiates a cascade of signals, culminating in the phosphorylation of eukaryotic initiation factor 2α which slows the rate of translational initiation (Weiler et al., 1996; Reilly et al., 1998; reviewed in Brostrom and Brostom, 1998). Recently, Biswas and co-workers (1999) proposed that mitochondrial stress signals are transduced to the nucleus via Ca^{2+} signals, a mechanism that is reminiscent of the transduction of ER stress signals to the nucleus (Pahl and Baeuerle, 1997).

Farther down the secretory path, protein sorting, transport and packaging are also influenced by Ca^{2+} (particularly lumenal Ca^{2+}, $[Ca^{2+}]_L$). Studies have shown that the exit of newly folded proteins from the ER (Lodish and Kong, 1990) and their transport between the ER and Golgi (Beckers and Balch, 1989) are inhibited by treatments that discharge ER Ca^{2+} stores. Intact Ca^{2+} homeostasis in the ER and Golgi is required for post-translational processing, including the proteolytic cleavage of many hormone precursors such as proinsulin and prosomatostatin (Di Jeso et al., 1997; Guest et al., 1997; Austin and Shields, 1996). Some of the Ca^{2+}-regulated protein processing and folding may be mediated through ER lumenal proteins, such as calreticulin and immunoglobulin binding protein (BiP), that do 'double-duty' as chaperone proteins and high-capacity Ca^{2+} buffering proteins (Krause and Michalak, 1997; Corbett et al., 1999; Lièvremont et al., 1997). Decreasing $[Ca^{2+}]_L$ may also accelerate protein degradation (Wileman et al., 1991) and cause the loss of resident ER proteins (Booth and Koch, 1989). Elevated $[Ca^{2+}]_L$ and acidic pH are required for sorting hormones and their processing enzymes into secretory granules of the regulated secretory pathway (Canaff et al., 1996; Song and Fricker, 1995). It is possible that certain hormones may differentially aggregate at specific Ca^{2+} concentrations. If this were found to be typical, it would suggest that local Ca^{2+} gradients in the *trans* Golgi compartments may facilitate preferential sorting of hormones into specific granule populations. In neuroendocrine cells that release multiple peptide hormones, this may be of particular importance. For example, differential sorting has been reported for luteinizing hormone and follicle-stimulating hormone, which are known to be independently regulated during some parts of the rat reproductive cycle (Thomas and Clarke, 1997; Childs et al., 1987).

Prior to exocytosis, several reactions such as granule transport, docking and priming may also be regulated by Ca^{2+} in several cell types (Neher and Zucker, 1993; Smith et al., 1998; Y. A. Chen et al., 1999). It is generally thought that these reactions occur prior to, and do not obligate, the final step of membrane fusion. In this case, moderate Ca^{2+} elevations may modulate priming, while exocytosis may require higher local $[Ca^{2+}]$ (Neher, 1998; Y. A. Chen et al., 1999). Therefore, it is likely that these steps in the secretory pathway are specifically modulated by the spatial segregation and amplitude of Ca^{2+} signals.

Several novel developments in the study of Ca^{2+}-triggered exocytosis have recently been reported. A general scheme for the control of exocytosis by localized micromolar Ca^{2+} domains has been widely accepted and these fundamental considerations have been reviewed elsewhere (Kasai, 1999; Neher, 1998; Südhof, 1995). One new area of interest centers around the possible differential involvement of Ca^{2+} in several distinct modes of secretion. Although it is clear that Ca^{2+} signals trigger agonist-evoked exocytosis, much less is known about the involvement of Ca^{2+} in the basal secretion that is prominent in many neuroendocrine cell types, and may be controlled independently (see, for example, Chang et al. 2000). Basal release can arise from the constitutive/constitutive-like secretory pathways or a steady leak from a pool of secretory granules in the regulated pathway (Burgess and Kelly, 1987; Kuliawat and Arvan, 1992). Although a dramatic elevation of $[Ca^{2+}]_c$ is not required for basal exocytosis in some neuroendocrine systems (Masumoto et al., 1995; see Chapter 7), Ca^{2+} levels in the lumen of secretory granules ($[Ca^{2+}]_{SG}$) could conceivably play a role in hormone release under unstimulated conditions. Indeed, constitutive homotypic membrane fusion of yeast vacuole is mediated by Ca^{2+} released from internal stores acting through CaM (Peters and Mayer, 1998). CaM and PKC have been shown to enhance the sensitivity of the secretory apparatus to existing Ca^{2+} levels (Y. A. Chen et al., 1999). These data form the basis for the hypothesis that basal secretion in neuroendocrine systems may result from secretory granules that carry with them sufficient Ca^{2+} that, when released in a highly localized manner, promotes their exocytosis. Such a basal release pathway could be independent of large-scale changes in $[Ca^{2+}]_c$ and could, theoretically, be modulated by agents that reduce basal hormone release.

Another very interesting and novel topic is the post-fusion regulation of exocytosis in the regulated secretory pathway (Rahaminoff and Fernandez, 1997). Evidence from morphological, electrophysiological and amperometry studies indicate that secretory material can be released without complete fusion of the secretory granule in a variety of cell types, including chromaffin cells and pancreatic β-cells (Rahaminoff and Fernandez, 1997; Zhou and Misler, 1996). This ultra-fast secretory granule recycling process bypasses the classical, clathrin-mediated endocytosis pathway. The probability of this so called "kiss-and-run" exocytosis increases with high Ca^{2+} and can be modulated by hormones (Alés et al., 1999; Angelson et al., 1999). In addition to increasing the frequency of "kiss-and-run" exocytosis, Ca^{2+} has the additional effect of alkalinizing the lumen of the secretory granules, thereby facilitating the 'leak' of peptide contents into the extracellular space through an open or partially open fusion pore (Han et al.,

1999). Taken together, these results also suggest that regulation of $[Ca^{2+}]_c$ or $[Ca^{2+}]_{SG}$ may be a target for treatments that inhibit basal hormone release. These findings likely represent only a first glimpse at numerous novel and physiologically important mechanisms for regulating hormone and transmitter release.

Current evidence strongly indicates that Ca^{2+} is involved in most facets of the secretory pathway, from gene transcription to post-fusion regulation of exocytosis. It is apparent that the secretory pathway is exquisitely sensitive to alterations in Ca^{2+} homeostasis, especially in the lumenal compartments. At present, many of the findings concerning Ca^{2+} regulation of different parts of the extended secretory pathway are difficult to reconcile. For example, further work is required to reconcile the dilemma that gene expression and exocytosis stimulated by many agonists seems to deplete intracellular Ca^{2+} stores, a maneuver that may generally result in a reduction of translational initiation and protein processing. There is an urgent need for studies that measure multiple Ca^{2+}-dependent cellular functions, as well as, the Ca^{2+} signals in multiple compartments/subcompartments, all in spatial and temporal detail. Depending on what these studies find, there may be a need to examine the problem in the context of multiple function-specific Ca^{2+} stores (see Section II). The use of well-characterized physiological agonists will also be important, because agents like ionomycin and thapsigargin may simultaneously deplete several classes of Ca^{2+} stores, while preventing the normal sequestration and cycling of Ca^{2+}. Manipulations that mimic the unique spatial and temporal features of agonist-evoked Ca^{2+} signals are needed to circumvent the potential pitfalls associated with non-selective Ca^{2+} drugs. For example, Ca^{2+} oscillations common to many neuroendocrine regulators may strike a balance, in time and space, between the alternative needs for 1) Ca^{2+} required for protein processing and 2) Ca^{2+} needed to trigger exocytosis.

It is clear that very complex Ca^{2+} signalling strategies are required to specifically regulate different elements of the extended secretory pathway, which can be either functionally coupled or independent of each other. This problem is confounded by the numerous other Ca^{2+}-sensitive activities that progress simultaneously in living cells. The obligatory complexity and specificity in Ca^{2+} signalling may be imparted through strategies that involve the spatial and/or temporal coding of Ca^{2+} signals made possible by the heterogeneity of Ca^{2+} stores and influx pathways found in many cell types.

Section II: Heterogeneity of intracellular Ca^{2+} stores and influx pathways

One possible solution to the problem of selective control over multiple Ca^{2+}-regulated cellular processes is to have multiple Ca^{2+} sources, each with distinct Ca^{2+} release and uptake dynamics, that can act alone or in combination to code for specific outcomes (Fig. 1.1). This section will explore this possibility. Relatively little is known about the contributions of multiple Ca^{2+} stores to the differential regulation of cellular functions. In the past, researchers considered only one type of intracellular Ca^{2+} store in most cell types. However, multiple distinct intracellular Ca^{2+} stores are generally revealed in systematic studies.

These include, but are not limited to, the ER, nucleus, mitochondria, Golgi, secretory granules and the cytosol proper (Pozzan et al., 1994; Pezzati et al., 1997; Tanaka and Tashjian, 1993; Fig. 1.2). At least five pharmacologically distinct Ca^{2+} pools have been assigned to the ER alone, if one considers evidence across cell types (Fig 1.1). It is likely that multiple distinct subcompartments with unique functional properties also exist within the other Ca^{2+}-storing organelles.

The cytosol as a determinant of Ca^{2+} signalling

One of the most important, and often overlooked, Ca^{2+} signalling components in the cell is the cytosol proper. The cytosol has remarkable Ca^{2+} buffering properties and is the prime determinant of the spatial and temporal characteristics of Ca^{2+} signals (Allbritton et al., 1992; Clapham, 1995; See Section III). Generally, free Ca^{2+} accounts for less than 1% of the total cytosolic concentration, although some variability exists in the values obtained from different cell types (Tse et al., 1994; Neher and Augustine, 1992; Clapham, 1995; Carafoli, 1987). It is interesting to consider the possibility that cytosolic Ca^{2+} buffering capacity may be regulated hormonally. It is also likely that subtle differences exist in the cytosolic buffering properties between cell types. Notwithstanding, it is clear that the ability of cells to maintain a low resting $[Ca^{2+}]_c$ is critical for survival as well as signalling.

Ca^{2+} can be pumped out of the cytosol by several mechanisms, including a multi-gene family of plasma membrane Ca^{2+}ATPases (PMCA) and Na^+/Ca^{2+} exchange (NCX; Carafoli, 1992). The relative importance of PMCA and NCX is cell type-dependent (Monteith and Roufogalis, 1995). Although active under basal conditions, the Ca^{2+} efflux mechanisms can be regulated by agonists through many endogenous effectors (Monteith and Roufogalis, 1995). The PMCA is stimulated directly by CaM, as well as phosphorylation by PKA or PKC (Carafoli, 1992; Monteith and Roufogalis, 1995).

Multiple extracellular Ca^{2+} influx pathways and their effects on intracellular Ca^{2+} stores

Cells harness the massive gradient of free Ca^{2+} between the cytosol and the extracellular space to generate fast and discrete signals. Influx of extracellular Ca^{2+} is mediated by a suite of voltage-dependent and -independent channels (reviewed in Tsien and Tsien, 1990). Of these, voltage-gated Ca^{2+} channels (VGCC) are best understood. Several classes of VGCC may contribute to agonist- and function-specific Ca^{2+} signals. How plasma membrane oscillators lead to regular fluctuations in $[Ca^{2+}]_c$ has received considerable experimental and theoretical attention. Several excellent reviews on this topic exist and the reader is directed to these articles (Sneyd et al., 1995; Petersen et al., 1994; Fewtrell, 1993).

Extracellular Ca^{2+} influx and efflux from intracellular stores are functionally intertwined in many ways. Ca^{2+} influx is required to refill agonist-sensitive Ca^{2+} stores in excitable and non-excitable cells. In most neuroendocrine cells studied to date, pharmacological or hormonal depletion of inositol 1,4,5-trisphosphate (IP_3)- or caffeine-sensitive intracellular Ca^{2+} stores leads to the

activation of an inward cation current (Ca^{2+} specific in most cell types) through a group of channels known collectively as store-operated Ca^{2+} channels (SOC) that remain only partially characterized (Putney and McKay, 1999; Bennett et al., 1998; Fomina and Nowycky, 1999). Perhaps the best-characterized store-dependent conductance is the I_{CRAC} of macrophages and lymphocytes. Several candidate proteins have been proposed to mediate analogous currents (Xu et al., 1997; Vannier et al., 1999; Montell, 1997; Putney, 1999a; Putney, 1999b). The mechanism by which the stores transmit their 'empty' signal to the plasma membrane is controversial (Clapham, 1995; Putney and McKay, 1999). Two ideas, the involvement of a Ca^{2+}-influx factor generated at the ER and the direct physical interaction of ER Ca^{2+}-release channels with store-operated channels, have received the most attention. Although, several major advances in this area have been achieved, much more work is required before the complexity and diversity of store-operated Ca^{2+} pathways can be understood.

Regardless of the influx mechanism, it is clear that the filling state of intracellular Ca^{2+} stores can be highly dependent on extracellular Ca^{2+} availability. Exposure of cells to nominally Ca^{2+}-free conditions leads to the abrupt depletion (within several minutes) of rapidly exchanging intracellular stores (Tse et al., 1993; Van Goor et al., 1999). Thus, many conclusions that excluded the involvement of intracellular Ca^{2+} stores, based solely on the requirement of extracellular Ca^{2+}, may need to be revisited. It is obvious that Ca^{2+} entry through VGCC or SOC plays a vital, permissive and often paramount role in cellular functioning, especially in certain cell types. The regulation of $[Ca^{2+}]_c$ by VGCC and other influx mechanisms has been thoroughly reviewed elsewhere (Berridge, 1998; Tsien and Tsien, 1990). Intracellular Ca^{2+} stores are ubiquitous and have been found to participate in a great number and variety of cell functions (Pozzan et al., 1994). The following sub-sections of this chapter will focus primarily on the role of Ca^{2+} released from organelles such as the ER, nucleus, mitochondria, Golgi, and secretory granules, and how these release processes may function at the molecular level.

The endoplasmic reticulum as a prototypical heterogeneous Ca^{2+} store

The importance of intracellular stores in Ca^{2+} signalling and basal Ca^{2+} homeostasis is now recognized for both excitable and non-excitable cells (Clapham, 1995; Berridge, 1998; Svoboda and Mainen, 1999). Chief among intracellular Ca^{2+} stores, in terms of proposed physiological importance, is the ER (including the sarcolasmic reticulum for the purposes of this thesis). The general principles of ER-mediated Ca^{2+} signalling have been reviewed elsewhere (Pozzan, 1994; Tsien and Tsien, 1990; Clapham, 1995). In this chapter, we will focus on recent advances and concepts that are of particular importance to physiologists and neuroendocrinologists.

The ER Ca^{2+} concentration has been estimated to be in the millimolar range using targeted aequorin (Montero et al., 1997), compartmentalized Ca^{2+} indicator dyes (Golovina and Blaustein, 1997), and electron energy loss imaging (Pezzati et al., 1997). One of the most significant findings of these studies is that the Ca^{2+} levels in the ER were found to be heterogeneous. For example, Golovina

and Blaustein (1997) imaged spatially and pharmacologically distinct ER Ca^{2+} stores in intact astrocytes and myocytes. Similarly, intact PC12 and HeLa cells possess both high [Ca^{2+}] and low [Ca^{2+}] ER subcompartments (Montero et al., 1997; Pezzati et al., 1997). The ER fractions of neuroendocrine PC12 cells also display considerable heterogeneity in the distribution of Ca^{2+} release channels, Ca^{2+} pump isoforms and Ca^{2+} buffering proteins (Rooney and Meldolesi, 1996). Taken together, these reports suggest that spatial, pharmacological and functional heterogeneity may be a common feature of the ER in all cell types.

The most common intracellular Ca^{2+}-sequestering mechanisms are ATP-dependent transporters. The ubiquitous sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) family of genes encode five SERCA protein isoforms with different functional properties. The constitutive activity of SERCA is a major factor in cytoplasmic and ER Ca^{2+} homeostasis. All known SERCA isoforms are sensitive to inhibition by thapsigargin (Lytton et al., 1991). The activity of SERCA can be modulated by hormones, including insulin (Xu et al., 1999). Additional forms of ATP-dependent, thapsigargin-resistant Ca^{2+} transport may exist on a number of Ca^{2+}-storing organelles, including ER (see below; Pizzo et al., 1997; Tanaka and Tashjian, 1993).

As is the case in cytosol, the lumen of the ER contains abundant Ca^{2+} buffering proteins. For example, calsequestrin and calreticulin, in muscle and non-muscle cells, respectively (Kawasaki and Kasai, 1994; Krause and Michalak, 1997), reduce the effective Ca^{2+} gradient that SERCAs work against. These proteins are important for many aspects of Ca^{2+} homeostasis in the lumen of the ER and other Ca^{2+} stores, and thus may affect Ca^{2+} signalling (Lleyelyn et al., 1998; Krause and Michalak, 1997). Overexpression of calreticulin has been shown to inhibit agonist-stimulated Ca^{2+} signals in *Xenopus* oocytes (Camacho and Lechleiter, 1995). It has also been suggested that calreticulin may directly modulate the activity of SERCA (John et al., 1998). Taken together, these studies demonstrate that lumenal Ca^{2+} binding proteins can have diverse roles in cell functioning, aside from the direct and indirect modulation of cytosolic Ca^{2+} signals.

Two types of widely expressed ER Ca^{2+}-release channels have been studied in detail. Both the IP_3R and the ryanodine receptor (RyR) belong to families encoded by three known genes, although additional isoforms of both proteins are produced by alternative splicing (Pozzan et al., 1994; Philips et al., 1996). The IP_3R genes and RyR genes encode related products that form large highly-conserved tetramers (300 and 550 kDa; Berridge, 1997; Pozzan et al., 1994). The most important functional commonality is that both the IP_3R and RyR Ca^{2+} channels are opened by Ca^{2+}; their respective second messengers, IP_3 and cyclic adenosine 5'-diphosphate ribose (cADPR), modulate the Ca^{2+}-activation threshold (Bezprozvanny et al., 1991; Berridge, 1997). IP_3 activates its receptor channel by relieving the inhibitory effects of high [Ca^{2+}] (Mak et al., 1998). Although the IP_3R and RyR proteins are functionally similar, there are important kinetic differences which may underlie the diversity of Ca^{2+} signals generated by these channels. For example, the IP_3R and the RyR differ with respect to their conductance, with the latter being greater (Bezprozvanny et al., 1991). Although

these two Ca^{2+}-mobilizing pathways share many common features, the functional differences discussed below may provide the basis for function- and agonist-specific Ca^{2+} signalling.

The IP_3 receptor signalling system

The signalling cascade initiated by agonist activation of heterotrimeric GTP-binding protein ($G_{q/11}$) stimulation of phospholipase C (PLCγ), which results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP_3 and 1,2-diacylglycerol (DAG), has been thoroughly studied in many cell types (Clapham, 1995; Petersen et al., 1994; Berridge, 1987). This reaction represents a major bifurcation in the signal transduction of extracellular regulators, as DAG is an endogenous activator of PKC (Berridge, 1987). IP_3 is a highly diffusible second messenger that binds to the IP_3R Ca^{2+} channel on intracellular membranes. IP_3 is metabolized by trisphosphatase into inositol 1,4-bisphosphate, which does not have Ca^{2+}-releasing properties (Berridge, 1987). Although IP_3 metabolism also results in the formation of other inositol phosphates which are thought be physiologically important, the actions of IP_3 are by far the best understood.

Three sub-types of the IP_3R have been characterized. The relative abundance of these proteins is controlled in a developmentally-regulated and tissue-specific manner, suggesting distinct physiological roles for each subtype, or combination thereof (Miyakawa et al., 1999; Wojcikiewicz, 1995). Accordingly, the known IP_3R isoforms display important functional differences. Individual IP_3R subtypes, and possibly other heterotetrameric isoforms, differ with respect to their Ca^{2+} and ATP sensitivity (Miyakawa et al., 1999; Ramos-Franco et al., 1998). Based on single-channel studies of the IP_3R1 in lipid bilayers, the IP_3R channel was thought to have a 'bell-shaped' sensitivity to [Ca^{2+}] that peaks with maximal stimulation at ~300 nM Ca^{2+} (Bezprozvanny et al., 1991; Mak et al., 1998). In contrast, the IP_3R2 and IP_3R3 are not inhibited by high [Ca^{2+}] (Hagar et al., 1998; Ramos-Franco et al., 1998). These findings suggest that the IP_3R2 and IP_3R3 release more Ca^{2+} *in vivo*, because their activity is not self-limiting. Experimental evidence suggests that the IP_3R3 mediates the initial amplification step that triggers Ca^{2+} signals in a number of cell types (Hagar et al., 1998; discussed in Section III).

Another characteristic property of the IP_3R family is spontaneous activity (Pozzan et al., 1994). This corresponds to the spontaneous Ca^{2+} release from IP_3-sensitive Ca^{2+} stores (Missiaen et al., 1991) that, together with a passive leak, can be measured when SERCA inhibitors are applied to cells. The activity of these channels can be stimulated through phosphorylation by PKA (Islam et al., 1998; Bird et al., 1993; Liu et al., 1996), and to a lesser extent PKC, CaM and members of the Src family of non-receptor tyrosine kinases (Jayaraman et al., 1996). It is currently unclear what role these phosphorylation events play in spontaneous- or agonist-generated Ca^{2+} signals from IP_3-sensitive Ca^{2+} stores.

Ryanodine receptor and cADP-ribose Ca^{2+} signalling

Intracellular Ca^{2+} stores containing a receptor/channel sensitive to ryanodine (RyR) were originally thought to reside exclusively in muscle cells. However, the presence of the RyR and its involvement in Ca^{2+} signalling is now

appreciated for many non-muscle tissues and represents a major IP_3-insensitive Ca^{2+} store found in many cell types (Pizzo et al., 1997; Sorrentino and Volpe, 1993; Pozzan et al., 1994). Ryanodine-sensitive Ca^{2+} stores are considered to be spatially and functionally distinct from those sensitive to IP_3 (Golovina and Blaustein , 1997). Three RyR isoforms are known and, like the isoforms of the IP_3R, may have important functional differences and tissue specificity (Bezprozvanny et al., 1991). Skeletal (RyR1), cardiac (RyR2) and brain (RyR3) isoforms are all found in the CNS (Sorrentino and Volpe, 1993), but only RyR2 and RyR3 are found in the rat pituitary (Sundaresan et al., 1997). Ryanodine specifically blocks all forms of the RyR at micromolar concentrations, whereas nanomolar ryanodine activates the channel by stabilizing it in a sub-conductance state (Ehrlich et al., 1994). Ryanodine binds to the RyR in its open conformation, suggesting its effects may be use-dependent. Millimolar concentrations of caffeine, which sensitize the RyR to Ca^{2+}, are often used as a probe for its activity.

Like the IP_3R, the RyR is activated by Ca^{2+}. Unlike the IP_3R1, elevations above resting levels of Ca^{2+} are stimulatory at most physiological concentrations. On the basis of its activation by physiological levels of Ca^{2+}, the RyR was ascribed a primary role in propagating Ca^{2+} waves through the process of Ca^{2+}-induced Ca^{2+}-release (CICR). At this time, it is not clear how CICR terminates or how Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores can be graded, since one would expect CICR to be prone to regenerative behavior. Several schemes, including 'adaptation', store depletion and local inactivation have been proposed to explain the observed refractoriness of RyR-mediated Ca^{2+} release (Györke and Fill, 1993; Sham et al., 1998; Stern, 1996). The activity of these channels can also be positively and negatively regulated by phosphorylation (Islam et al., 1998; Wang and Best, 1992). Notably, the RyR can be inhibited by CaMK-II (Wang and Best, 1992) which could provide a long-term means for modulating the duration of regenerative RyR-mediated Ca^{2+} signals.

The CICR function of ryanodine-sensitive Ca^{2+} stores has been studied in detail. It is now clear that the RyR can also be a primary target for receptor-generated Ca^{2+}-mobilizing chemical messengers (e.g. Tanaka et al., 1998). The endogenous effector for the RyR2 and RyR3 isoforms is cADPR (Mészáros et al., 1993). This novel second messenger can be generated by the ubiquitously distributed enzymes, ADP-ribosyl cyclase and CD38 (Galione and White, 1994; Pozzan et al., 1994; Kato et al., 1999). cADPR evokes Ca^{2+} signals in a variety of cell types, including pituitary-derived GH_4C_1 cells (Koshiyama et al., 1991). Other second messengers may also target the RyR, including sphingosine-1-phosphate (S1P), 2'-phospho-cADPR and acyl-coenzyme A (Choi et al., 1996; Vu et al., 1996; Fitzsimmons et al., 1997 and references therein).

cADPR has been proposed as a possible link between nitric oxide and downstream Ca^{2+} release through the RyR (Galione and White, 1994). These downstream actions may play a role in several cellular processes including long-term synaptic depression in neurons (Reyes-Harde et al., 1999). Nitric oxide also elicits Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores in pancreatic β-cells (Willmott et al., 1995). These actions may be mediated by cGMP, which is known

to participate in Ca^{2+} homeostasis in a number of cell types (Hajnóczky et al., 1994). When viewed together, these reports indicate that RyR-mediated Ca^{2+} release is an important component in several signal transduction pathways. The degree to which RyR and IP_3R Ca^{2+} signals control different cell functions within individual cell types remains to be elucidated.

Nicotinic acid adenine dinucleotide phosphate-sensitive Ca^{2+} stores

Another pyridine nucleotide-derived second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP), has also been studied for its role in releasing Ca^{2+} from intracellular stores (Lee, 2000). Like cADPR, NAADP is produced by ADP-ribosyl cyclase (Genazzani and Galione, 1997). NAADP mobilizes Ca^{2+} from a compartment that is separate from those sensitive to IP_3, cADPR and thapsigargin, although ATP is still required for its refilling (Genazzani and Galione, 1997; Genazzani and Galione, 1996). Surprisingly, the putative NAADP-gated intracellular channel is not modulated by $[Ca^{2+}]_c$, indicating that it does not mediate CICR (Chini and Dousa, 1996). This finding contrasts with all other intracellular Ca^{2+} channels studied to date, and suggests that the NAADP receptor may play a unique role in shaping Ca^{2+} signals. Recently, a physiological role for the NAADP Ca^{2+} release system in pancreatic acinar cells has been proposed. This report demonstrated that cholecystokinin uses NAADP-sensitive Ca^{2+} stores as the initial trigger for Ca^{2+} signals that are subsequently transduced by IP_3R- and RyR-dependent mechanisms (Cancela et al., 1999). Given the ubiquity of the NAADP-generating enzymes, it is likely that agonist-evoked Ca^{2+} release from NAADP-sensitive stores may be common. Interestingly, NAADP-activated Ca^{2+} release from intracellular stores is blocked by diltiazen, nifedipine, BAY K 8644 and verapamil, all classical inhibitors of L-type VGCC (Genazzani Galione, 1997). Since these drugs are well known blockers in many neuroendocrine systems, we may have already, unknowingly, obtained evidence of extensive NAADP involvement.

Sphingolipids and metabolites

Sphingolipids are important components of eukaryotic cell membranes that have been implicated in intracellular Ca^{2+} homeostasis and signalling (Zhao et al., 1994; Choi et al., 1996). Certain G-protein-coupled receptors activate sphingosine kinase (Heringdorf et al., 1998) leading to the production of sphingosine 1-phosphate (S1P) and the subsequent mobilization of Ca^{2+} from IP_3-independent ER stores which, in some cases, may be Ry-sensitive (Choi et al., 1996; Tas and Koschel, 1998). Interestingly, S1P can be produced at the ER membrane (Ghosh et al., 1994) suggesting the possibility of localized signalling. The putative ER-derived signals could also mediate communication with other parts of the cell. In agreement with this hypothesis, sphingolipids have been implicated in the regulation of SOCs (Mathes et al., 1998).

S1P is not the only Ca^{2+}-mobilizing sphingolipid. Others have reported IP_3-independent Ca^{2+} mobilization by sphingosyl-phosphocholine (SPC) and not S1P in certain cells (Mao et al., 1996; Yule et al., 1993). These effects may be partially mediated by the RyR2, as is the case in cardiac muscle (Betto et al.,

1997). Notwithstanding, the product of the mammalian SCaMPER (sphingolipid Ca^{2+} release-mediating protein of endoplasmic reticulum) gene encodes a novel protein, unrelated to either the IP_3R or RyR, that mediates SPC-evoked Ca^{2+} release from ER stores (Mao et al., 1996). Taken together, these reports suggest the possibility that there may be at least two distinct sphingolipid-sensitive intracellular Ca^{2+} stores.

Evidence discussed thus far clearly indicate that multiple functional Ca^{2+} stores can coexist and suggests that these stores may regulate distinct cellular functions by producing kinetically and spatially unique Ca^{2+} signals. The question of how multiple functional Ca^{2+} stores are segregated within the ER, which is continuous by many accounts, is particularly intriguing (Golovina and Blaustein, 1997; Montero et al., 1997). However, it is conceivable that standing gradients in lumenal Ca^{2+}, similar to those already appreciated in the cytosol, may exist. Moreover, the structure of the ER is dynamic and actively regulated by Ca^{2+} (Subramanian and Meyer, 1997).

It must be noted that the participation of non-ER Ca^{2+} stores in many of the novel Ca^{2+} signalling systems described above cannot be excluded. Most often these possibilities have not been examined. Determining the anatomical identity of the Ca^{2+} stores mobilized by the second messenger systems noted above is not only expected to yield information regarding their physiological role, but also will undoubtedly be a fruitful and surprising area of inquiry in the future. In addition to sub-compartments of the ER, several candidate Ca^{2+}-regulating organelles, such as the Golgi, secretory granules, nucleus and mitochondria can be considered. These novel Ca^{2+}-storing organelles are the subject of the following sub-sections.

Golgi-mediated Ca^{2+} signalling

Like the ER, the Golgi stores large amounts of cellular Ca^{2+} and is an important regulator of Ca^{2+} homeostasis in many cell types, from yeast to mammalian cells (Pezzati et al., 1997; Miseta et al., 1999; Pozzan et al., 1994). Ca^{2+} uptake into the Golgi may occur via thapsigargin-sensitive and -insensitive mechanisms (Pinton et al., 1998; Taylor et al., 1997). The primary candidate for this thapsigargin-insensitive Ca^{2+}-ATPase is the homologue of the yeast Golgi P-type ATPase *PMR1* (Gunteski-Hamblin et al., 1992; Sorin et al., 1997; Pinton et al., 1998). Interestingly, mutants of *Saccharomyces cerevisiae* deficient in *PMR1* have impaired secretory function, suggesting a conserved role for Ca^{2+} homeostasis in the processing of secreted proteins (Rudolph et al., 1989). Experiments using HeLa cells expressing specifically targeted aequorin have shown that the Golgi contains an agonist-sensitive Ca^{2+} store that mediates Ca^{2+} release through the IP_3R1 (Pinton et al., 1998; Lin et al., 1999). However, the existence of novel Ca^{2+}-sequestering mechanisms, and the expression of Ca^{2+}-binding proteins that are found predominantly or exclusively in the lumen of the Golgi, suggest that the functional properties of this Ca^{2+} store are unique (Lin et al., 1999; Lin et al., 1998; Scherer et al., 1996). Localized Ca^{2+} signals could play a dramatic role in Golgi function, without significantly altering $[Ca^{2+}]_c$. Taken together, these observations demonstrate that the Golgi may be a physiologically

important Ca^{2+} store that may generate agonist- or function-specific Ca^{2+} signals. Since the Golgi contains IP_3- and thapsigargin-sensitive components, it is probable that some previous investigations have mistakenly attributed Golgi-evoked Ca^{2+} signals to the ER.

Ca^{2+} signalling in and around the secretory granules

Several lines of evidence suggest that secretory granules, which contain high concentrations of Ca^{2+}, may be the ultrastructural correlates of at least two novel classes of intracellular Ca^{2+} stores (Pezzati et al., 1997; Pozzan et al., 1994). First, many cell types, including pituitary cells, contain a slowly-exchanging Ca^{2+} store that can be released by treatments that collapse pH gradients of acidic organelles, but is insensitive to ionomycin and a number of IP_3-generating hormones (Martinez et al., 1996; Shorte et al., 1991; Pozzan et al., 1994). However, this type of secretory granule Ca^{2+} store has not been studied extensively enough to exclude its participation in agonist or antagonist regulation in all neuroendocrine systems.

There is increasing evidence that a second type of secretory granule can be an IP_3- or agonist-sensitive Ca^{2+} store in many cell types, including endocrine (β and δ) and exocrine cells of the pancreas, tracheal goblet cells and *Aplysia* neurosecretory cells (Blondel et al., 1994; Gerasimenko et al., 1996a; Nguyen et al., 1998; Jonas et al., 1997). Nguyen and coworkers (1998) recently demonstrated that IP_3 generated oscillations in a subset of secretory granules from rabbit trachea that preceded, and were shown to drive, cytosolic Ca^{2+} fluctuations. Taken together, these reports suggest that the secretory granules may be more important than previously appreciated in the transduction of certain stimuli in some cell types. As is the case with the Golgi Ca^{2+} stores, many past studies might have incorrectly attributed secretory granule Ca^{2+} release to the ER.

Ca^{2+} signalling in the nucleus

Many Ca^{2+}-dependent cellular functions are compartmentalized within the nucleus. Although the understanding of nuclear Ca^{2+} signalling is clearly of great importance, it is poorly characterized at present. Specifically, whether the nucleus represents a discrete Ca^{2+} store, or is functionally continuous with the cytosol is still a contentious issue. Although it has been assumed that the nuclear pore complex would freely pass small molecules, there are now many reports of differential regulation of nuclear and cytoplasmic Ca^{2+} using a variety of cell types and measurement strategies (reviewed in Rogue and Malviya, 1999; Korkotian and Segal, 1996; Badminton et al, 1996; Al-Mohanna et al., 1994). The nucleus can generate IP_3 locally. It also possesses the components required to regulate Ca^{2+} independently from the cytosol, including IP_3R, RyR and SERCA (Gerasimenko et al., 1996b). Still, some work has shown that nuclear Ca^{2+} is not maintained differentially from $[Ca^{2+}]_c$ (Rogue and Malviya, 1999). The discrepancies found in the literature may be due to cell type differences. A more likely explanation is that the degree to which nuclear $[Ca^{2+}]$ is coupled to, or insulated from, cytosolic Ca^{2+} signals is dynamic and under complex regulatory control. The finding that Ca^{2+} is involved in the normal functioning and

restructuring of the nuclear envelope (Stehno-Bittel et al., 1995; Baitinger et al., 1990; Subramanian and Meyer, 1997) supports such a hypothesis. Other aspects of nuclear Ca^{2+} signalling that also require clarification include the nature of nucleoplasmic Ca^{2+} homeostasis, the degree of continuity with ER Ca^{2+} stores, the role of the differential distribution of Ca^{2+} signalling proteins on the inner and outer nuclear membranes and the role of the nuclear pore complex (as well as the interactions between these components). The answers to these questions are bound to have important implications in the regulation of cell function.

Involvement of mitochondria in Ca^{2+} signalling

The capability of mitochondria to transiently sequester large quantities of Ca^{2+} has been appreciated for some time. It is now clear that mitochondria play a very important physiological role in the basal Ca^{2+} homeostasis and agonist-evoked Ca^{2+} signals (Babcock et al., 1997; Pozzan et al., 1994). Resting free intramitochondrial Ca^{2+} levels are only slightly greater than in the cytosol (Duchen, 1999). Ca^{2+} is sequestered, at the expense of generating ATP, by energized mitochondria through a uniporter that exploits the electrochemical proton gradient, and is rapidly lost through Na^+/Ca^{2+} exchange and other mechanisms (Duchen, 1999; Pozzan et al., 1994; Ichas et al., 1997). The Ca^{2+} uniporter of the mitochondria inner membrane is a high-capacity/low-affinity transport system, and therefore acts as a 'high-pass' filter, shaping the peak and decay phases of transient Ca^{2+} signals that reach micromolar amplitudes (reviewed in Duchen, 1999). This Ca^{2+} buffering activity of mitochondria has a profound effect on the spatial and temporal characteristics of Ca^{2+} signals in many cell types, including endocrine cells (Herrington et al., 1996; Hehl et al., 1996). In addition to modulating Ca^{2+} signals generated by extracellular Ca^{2+} influx through VGCC or SOC (Hoth et al., 1997), mitochondria have a dynamic and reciprocal interaction with ER Ca^{2+} release channels (Rizzuto et al., 1993; Rizzuto et al., 1998). Mitochondria can exert dual control on Ca^{2+} signals generated by intracellular Ca^{2+} stores. On one hand, Ca^{2+}-uptake may stabilize steady-state $[Ca^{2+}]_c$ by attenuating 'accidental' Ca^{2+} signals evoked by spontaneous activity of the IP_3R. Conversely, mitochondria can enhance IP_3R-mediated Ca^{2+} signalling by preventing Ca^{2+}-dependent autoinhibition through the sequestration of localized high amplitude Ca^{2+} events (Duchen, 1999). ER-mitochondria complexes have been implicated in the propagation of Ca^{2+} waves (Simpson et al., 1997; Csordás et al., 1999). Taken together, these findings indicate that the mitochondria play a primary role in regulating Ca^{2+} signalling.

Mitochondria, together with the other Ca^{2+}-storing organelles that have been discussed above, form a complex network for Ca^{2+} signalling. Their morphological and functional diversity allow cells to generate sufficiently complex Ca^{2+} signals in response to extracellular stimuli. Examples of the spatial and temporal complexity of Ca^{2+} signals, and how information encoded in these patterns may independently regulate specific cellular functions is discussed in the next section.

Section III: Spatial and Temporal Characteristics of Function- and Agonist-Specific Ca^{2+} Signalling

Mechanisms of spatially restricted Ca^{2+} signals

Up to this point, I have reviewed evidence that many functionally distinct Ca^{2+} stores can co-exist within single cells. Much of the value of multiple Ca^{2+} stores would be lost, however, if the Ca^{2+} signals could never remain independent in space. One of the most important determinants of Ca^{2+} signal formation is the capacity of the cytosol to act as a Ca^{2+} buffering medium (discussed above; Jafri and Keizer, 1995). Ca^{2+} signals are constrained spatially by the very low 'effective diffusion rate' for the ion in cytosol, when compared to IP_3, for example. Millimolar concentrations of immobile, and to a lesser extent mobile, Ca^{2+} buffering proteins bind Ca^{2+} and slow the diffusion rate of Ca^{2+} ions by over an order of magnitude (Allbritton et al., 1992; Tse et al., 1994). Endogenous calcium-binding proteins have low affinity and fast 'on-rates'; Ca^{2+} binding to endogenous buffers reaches equilibrium in less than 1 ms (Roberts, 1994; Naraghi et al., 1998; Jafri and Keizer, 1995). Ca^{2+} diffusion coefficients have been calculated for a number of cell types and the estimates are generally in agreement with one another. For example, Ca^{2+} diffuses at a rate of ~10 $\mu m^2/s$ in chromaffin cells (Zhou and Neher, 1993). However, this diffusion rate is dynamic with respect to ambient $[Ca^{2+}]$. In agreement, Allbritton and coworkers (1992) found that Ca^{2+} diffusion increases from ~13 $\mu m^2/s$ to ~65 $\mu m^2/s$ when $[Ca^{2+}]_c$ is changed from 90 nM to 1000 nM. This value is only a measure of the buffering capacity of the cytosolic Ca^{2+}-binding proteins, since it was obtained in *Xenopus laevis* oocyte cytosol without contribution of Ca^{2+} sequestering organelles, such as the mitochondria and ER. The effect of these components on Ca^{2+} diffusion is also dynamic and depends on the local $[Ca^{2+}]_c$ (Duchen, 1999; Pozzan, 1994). The physical presence of organelles can also have a large effect on the apparent Ca^{2+} diffusion rate (Naraghi et al., 1998). These properties of the cytosol have important consequences for the generation of Ca^{2+} signals.

Just as individual neurons in the brain must be able to fire independently, as well as in concert, the ability of cells to independently regulate Ca^{2+} in spatially distinct regions is a prerequisite for spatial coding of Ca^{2+} signals (discussed below). Can cells generate persistent, as well as transient, localized Ca^{2+} fluctuations in the continuous cytosolic space? Ca^{2+} gradients can arise when the rate of Ca^{2+} influx into a space exceeds the rate of diffusion out of that space (Machaca and Hartzell, 1999b). Since the diffusion of Ca^{2+} is comparably slow, steep local gradients may be common. Transient Ca^{2+} gradients of up to 100 μM do occur around the openings of Ca^{2+} channels or clusters of Ca^{2+} channels in both excitable and non-excitable cells. Ca^{2+} signals result from the coordinated recruitment of these elementary events, which are referred to as "Ca^{2+} sparks" or "Ca^{2+} puffs" (Berridge, 1997). Since the IP_3R and RyR are activated by Ca^{2+}, individual Ca^{2+}-release channels are engaged into the Ca^{2+} signal by the local diffusion of Ca^{2+} (Berridge, 1997). This process is facilitated by their respective second messengers. In many cell types, these events are initiated in 'trigger zones', which often have specific isoforms of the IP_3R or higher resting Ca^{2+} (Ito et al., 1997). For example, the specific properties of the IP_3R3 isoform are

favorable for initiating Ca^{2+} waves in the 'trigger zone' of pancreatic acinar cells (Nathanson et al., 1994; Thorn et al., 1993). The high resting [Ca^{2+}] in these regions also increases the Ca^{2+} signal gain in response to agonists. Once a Ca^{2+} signal is initiated, it can be regenerative in both time and space, much like an action potential (Berridge, 1998). Unlike an action potential, there is evidence that Ca^{2+} signals can be graded.

The propagation of Ca^{2+} signals often takes the form of a non-decremental wave. Two types of Ca^{2+} wavefronts, saltatory and continuous, have been recorded from a variety of cells types (Dawson et al., 1999). Rather than arising via different mechanisms, recent modeling work suggests that both types may originate through a common mechanism. An interesting prediction of the model proposed by Dawson and colleagues (1999) is that the propagation of Ca^{2+} waves is continuous if the release of Ca^{2+} from stores is rate-limiting, but saltatory if the diffusion from one release site to the next is rate-limiting. Given that the IP_3R and RyR (and their isoforms) have different unitary conductance, their relative distribution may dictate the type of Ca^{2+} signals generated by a specific cell or region of a cell.

As one might expect, the Ca^{2+} signalling machinery of the cell is distributed in a strategic manner within cells. In addition to the spatial heterogeneity of Ca^{2+}-storing organelles (Simpson et al., 1997), all types of Ca^{2+} channels and pumps on the plasma membrane or membranes of organelles have been shown to be spatially localized (Lee et al., 1997; Kasai et al., 1993). IP_3R and RyR are organized in clusters in a variety of cell types (Wilson et al., 1998; Mak and Foskett, 1997). Recent evidence suggests that this phenomenon can be acutely regulated by Ca^{2+} in rat basophilic leukemia cells (Wilson et al., 1998) suggesting that the mechanisms by which cells generate Ca^{2+} signals can be modulated by extracellular signals. The redistribution of Ca^{2+} signalling components could provide a general mechanism for regulating the excitability of neuroendocrine systems. In addition to regulating excitability of the intracellular Ca^{2+} signalling network, modifications to the routes by which Ca^{2+} waves travel could conceivably allow cells to switch the cellular functions that are regulated by a given stimulus. How cells control signal specificity by compartmentalizing function is the subject of the next sub-section.

Spatial compartmentalization of Ca^{2+}-dependent cellular functions

One method to ensure that localized Ca^{2+} signals regulate cellular functions with specificity is to spatially segregate the cellular functions. Spatial coding of Ca^{2+} signals is well established in neuronal models because of the obvious compartmentalization of cellular functions in the dendrites, soma, axon and synapse. There is ample evidence that this strategy is common to all cell types. Many Ca^{2+}-activated enzymes are compartmentalized or associated with specific membranes. Importantly, the spatial distribution of activated Ca^{2+} effector proteins, such as CaM and CaMK-II, reflect the spatial pattern of Ca^{2+} signals (Inagaki et al., 1997; Craske et al., 1999). This suggests that cells are capable of decoding spatial information.

Key regulators of cell excitability such as the Ca^{2+}-activated K^+ channel (Marrion and Tavalin, 1998) are known to exist in close proximity to Ca^{2+} channels, forming 'isolated' signalling complexes. Steep subplasmalemmal Ca^{2+} gradients, which are only detected with membrane targeted Ca^{2+} indicators, are correlated with the activity of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes (Machaca and Hartzell, 1999b). It was recently demonstrated that Ca^{2+} influx through SOC specifically regulates closely-associated Ca^{2+}-inhibitable adenylyl cyclase (Fagan et al., 1998).

Ca^{2+} signals can also be targeted to specific organelles without affecting the bulk cytosol. In many cell types, the mitochondria respond to localized, high-amplitude Ca^{2+} signals, delivered by closely positioned ER Ca^{2+} release channels, that are not observed in gross measurements of $[Ca^{2+}]_c$ (Rizzuto et al., 1998; Rizzuto et al., 1993). Ca^{2+} signals transmitted through the mitochondria are involved in metabolic pacing (Hajnóczky et al., 1995, Duchen, 1999) and steroid synthesis (Capponi et al., 1988). Spatial Ca^{2+} signalling may also be important in the differential regulation of gene expression. Ca^{2+} acts through at least two different signal transduction pathways to regulate hippocampal gene expression. Apparently, a CaM-mediated pathway is only required when Ca^{2+} influx occurs through VGCC, but not ionotropic *N*-methyl-d-aspartate receptors (Bading et al., 1993).

Spatial coding can also solve the problems associated with the independent regulation of the different sites on the extended secretory pathway, outlined in Section 1. The differential effects on gene expression, protein synthesis and protein processing can be reconciled by the functional compartmentalization of these functions in the nucleus, ER and Golgi, respectively. However, the solution to how the regulated and constitutive secretory pathways may be differentially controlled is less obvious. Some data support the possibility that the regulated and constitutive secretory pathways may be spatially segregated, in addition to having possible differences in their sensitivity to Ca^{2+}. Evidence for this comes from studies of neuroendocrine AtT-20 cells, and from the constitutive and regulated secretion of von Willebrand factor from human endothelial cells (Rivas and Moore, 1989; Sporn et al., 1989). These findings imply that localized Ca^{2+} signals can trigger the fusion of constitutive granules, without affecting the fusion of other cellular membranes.

There is growing evidence that spatially restricted exocytosis in neuronal active zones and polarized secretory cells are not exceptional cases and that regulated exocytosis can be spatially restricted in many cell types (Carabelli et al., 1998, Schroeder et al., 1994, Sporn et al., 1989). Colocalization of catecholamine secretion with subplasmalemmal Ca^{2+} domains has been directly observed in chromaffin cells (Robinson et al., 1995). On the other hand, a recent study using GFP-tagged clathrin demonstrated that spatially restricted endocytosis also occurs (Gaidarov et al., 1999). Taken together, these results suggest that many types of cells may compartmentalize Ca^{2+}-dependent secretion and, separately, membrane retrieval to avoid unwanted 'crosstalk' with other Ca^{2+}-dependent cellular functions.

The multiplicity of Ca^{2+} action suggests that inhibitory neuroendocrine regulators of hormone synthesis or release may also act at many potential target sites. For example, the lumens of Ca^{2+}-storing organelles may represent a potential site of neuroendocrine regulation of agonist-stimulation. Specifically, modulation of the lumenal Ca^{2+} environment in secretory granules would have marked effects on the secretion of hormones at both pre- and post-fusion stages (see Section I). Moreover, biochemically distinct populations of secretory granules could be targeted. Several lines of evidence suggest that many neuroendocrine inhibitors act at important sites late in the secretory pathway. For instance, the Ca^{2+}-dependent phosphatase, calcineurin, mediates the inhibition of insulin secretion by somatostatin, galanin and adrenaline through a mechanism that is distal to the depolarization-evoked Ca^{2+} signal (Renström et al., 1996). It is interesting to note that Ca^{2+}-dependent enzymes can both stimulate and inhibit secretion. Perhaps the unique Ca^{2+} signal leading to the activation of calcineurin is insufficient to activate Ca^{2+}-dependent pro-exocytosis signalling pathways. Further work is necessary before the complexity of Ca^{2+} involvement, both positive and negative, in the extended secretory pathway can be understood.

It is also interesting to consider the possibility of spatial heterogeneity of Ca^{2+} signals traveling within the lumens of organelles. The problems posed by the continuity of the lumen of the ER are analogous to those faced by the cytosol with respect to the creation of spatially localized Ca^{2+} signals. It is becoming clear that the cytosol uses the strategic and dynamic placement of Ca^{2+} pumps and channels to generate Ca^{2+} fluctuations with significant spatial and temporal complexity (Wilson et al., 1998; Lee et al., 1997; Kasai et al, 1993). This process is coupled to a very low diffusion rate for Ca^{2+} ion, and possibly the non-uniform distribution of buffering capacity in the cytosol. The lumen of the ER, which is heterogeneous in many respects, may use a similar strategy to transduce complex Ca^{2+} signals and modulate discrete lumenal targets. The spatial and temporal characteristics of lumenal Ca^{2+} signals may, in turn, have important roles in modulating cytosolic Ca^{2+} signals in a variety of cell types, as predicted by several theoretical and experimental studies (Jafri and Keizer, 1995; Lukyanenko et al., 1999; Nguyen et al., 1998). The possibility that complex Ca^{2+} signals in the lumen of Ca^{2+} stores may regulate cytosolic Ca^{2+} signalling in a reciprocal manner requires further attention.

Further research will be required to resolve the molecular mechanisms by which Ca^{2+} signals and Ca^{2+}-dependent cellular functions are colocalized, both in the cytosol and in organelle lumens. There is a potential role for 'scaffold' complexes, like those that regulate interactions of proteins in the MAPK pathways (Garrington and Johnson, 1999), in promoting the physical association of Ca^{2+}-binding and -signalling proteins with their targets. The cytoskeleton may also be prominently involved in the spatial aspects of Ca^{2+} signalling. There is evidence that the cytoskeleton mediates the coordination of Ca^{2+} mobilization between multiple Ca^{2+} stores (Hajnóczky et al., 1994) and the generation of agonist-evoked Ca^{2+} signals (Ribeiro et al., 1997). It is clear that the spatial complexity of Ca^{2+} signals is necessary to support the independent regulation of Ca^{2+}-dependent

cell functions. Equally complex are the temporal waveforms of agonist-generated Ca^{2+} signals, which are considered next.

Temporal coding of Ca^{2+} signals: Duration and amplitude

One important characteristic of physiological, high-amplitude Ca^{2+} signals, is that they must be transient to avoid Ca^{2+} cytotoxicity. Many factors shape the waveform of Ca^{2+} transients. The duration of Ca^{2+} signals may be controlled by specific characteristics of the IP_3R and RyR isoform(s) involved (detailed in Section II) and the rate of Ca^{2+} removal from the cytosol. In instances when prolonged signals are required, cells generally exhibit biphasic Ca^{2+} signals or elevations of Ca^{2+} with suitably low amplitude. The activation of protein kinases, such as CaMK also ensure that the influence exerted by extracellular signals on intracellular processes persists (MacNicol et al., 1990).

Notwithstanding, there are several notable examples where cell functions are controlled by the duration of Ca^{2+} signals *per se*. In T lymphocytes, NF-κB and Jun amino-terminal kinase are specifically stimulated by transient Ca^{2+} signals with high amplitude, while NFAT is activated by low-amplitude, long-duration Ca^{2+} signals (Dolmetsch et al., 1997). Recently, the translocation of PLA_2 from the cytosol to the nuclear envelope/ER and its subsequent activation were shown to be insensitive to brief Ca^{2+} transients, but is dependent on Ca^{2+} signals with a duration above 1 minute (Hirabayashi et al., 1999). Taken together, these reports suggest that the duration of Ca^{2+} signals can encode specific information.

The most obvious mechanism for regulating specific functions is by modulating the amplitude of Ca^{2+} signals. The range of affinities for Ca^{2+} shown by the suite of effector proteins found in cells extends over several orders of magnitude. This suggests that cells can selectively transduce information by targeting specific effector proteins using the amplitude of Ca^{2+} signals. Many examples of this are found in the literature. For example, micromolar Ca^{2+} signals were required to activate exocytosis in pancreatic acinar cells, while some membrane ion channels were specifically activated by lower amplitude Ca^{2+} transients, suggesting that the amplitude of the Ca^{2+} signal can encode specificity (Ito et al., 1997).

In addition, the amplitude of the steady-state Ca^{2+} levels can have profound effects on other signal transduction pathways. For example, whether the Na^+-K^+-ATPase is stimulated or inhibited by PKC or PKA depends on the local ambient $[Ca^{2+}]_c$ (Cheng et al., 1999). The resting Ca^{2+} concentration plays an important role in the generation of specific Ca^{2+} oscillations by agonists in pancreatic acinar cells (Toescu et al., 1993). Adjusting $[Ca^{2+}]_c$ slightly upwards increases the probability that CICR will occur. This effectively sets the cytoplasm closer to the threshold for Ca^{2+} signalling activity. Modulating the gain of the Ca^{2+} signalling system may be a common mechanism regulating cellular activity.

Temporal coding of Ca^{2+} signals: Oscillation frequency

Ca^{2+} oscillations are one of the most common Ca^{2+} signal waveforms seen in agonist-stimulated cells (Petersen et al., 1994). The ER membrane is excitable

and is capable of generating Ca^{2+} signals that are regenerative in both space and time (reviewed in Berridge, 1998). The mechanisms underlying Ca^{2+} oscillations have received much experimental and theoretical attention, and have been covered in several enlightening reviews (Fewtrell, 1993; Sneyd et al., 1995; Petersen et al., 1994). Some of these models incorporate Ca^{2+} stores as a single entity. However, since the evidence summarized above suggests that most cells possess multiple intracellular Ca^{2+} stores, each with distinct properties, the situation in living cells may be considerably more complex. In the rest of this section, I will describe recent advances in the understanding of the functional consequences of Ca^{2+} oscillations.

Ca^{2+} oscillations, and their frequency modulation, have been found to regulate many cellular functions. For example, in rat gonadotropes, each oscillation in $[Ca^{2+}]_c$ is associated with a burst of exocytotic activity (Tse et al., 1993). Ca^{2+} oscillation frequency is dose-dependent in many cells types (Woods et al., 1986; Petersen et al., 1994; Tse et al., 1993). Powerful evidence of Ca^{2+} signal coding through frequency comes from studies of Ca^{2+}-regulated gene expression. Dolmetsch and colleagues (1998) controlled the frequency of Ca^{2+} transients in T-lymphocytes by rapidly changing extracellular $[Ca^{2+}]$ while SOCs were kept open using thapsigargin. They demonstrated that high frequency oscillations activate the transcription factors, NF-AT, Oct/OAP and NF-κB, while lower frequency oscillations only activated NF-κB. Similar frequency-dependence of Ca^{2+}-regulated gene expression was also reported for rat basophilic leukemia cells challenged repeatedly with liberated 'caged IP_3' (Li et al., 1998). Moreover, Ca^{2+}-sensitive mitochondrial dehydrogenases are preferentially activated by Ca^{2+} oscillations in mitochondria, when compared to sustained Ca^{2+} elevations (Hajnóczky et al., 1995). Similarly, angiotensin II generates frequency-coded mitochondrial Ca^{2+} oscillations in adrenal glomerulosa cells that may be functionally important in aldosterone synthesis (Pralong et al., 1994).

Ca^{2+} oscillations on a larger time scale are also coded by frequency. For example, developing *Xenopus* neurons exhibit Ca^{2+} waves and spikes, in the growth cones and somata, respectively. The different frequencies of Ca^{2+} oscillations in these compartments lead to specific modulation of neurite outgrowth and differentiation (Gu and Spitzer, 1995). Pulsatility on a larger time scale is also common in neuroendocrine systems. For example, the frequency coding of fluctuations in gonadotropin-releasing hormone release from the hypothalamus, controls gonadotropin release and gene expression in the pituitary. As might be expected, pulses of elevated $[Ca^{2+}]_c$ are more effective than a sustained rise in regulating gene expression in gonadotropes (Haisenleder et al., 1997). Interestingly, the optimal pulse frequency was different between the two gonadotropic hormones, luteinizing hormone and follicle-stimulating hormone, suggesting a novel mechanism for their differential neuroendocrine regulation.

How are Ca^{2+} oscillations decoded? Illumination of this very important question has recently come from the elegant work of De Konink and Schulman (1998), who recently demonstrated that one of the major Ca^{2+} signal effector proteins, CaM kinase II, has the intrinsic property of being tuned to respond optimally to Ca^{2+} oscillations with higher frequency, *in vitro*. Others have found

that the translocation and accumulation of CaM in the nucleus of pancreatic acinar cells is best stimulated by agonist-generated Ca^{2+} oscillations (Craske et al., 1999). These results demonstrate, unambiguously, that Ca^{2+} effector proteins have the capacity to decode temporally complex Ca^{2+} signals with fidelity.

Temporal coding of Ca^{2+} signals: Rate of rise

Another temporal feature of Ca^{2+} signals that could encode information is their rate of rise, which has been considered important for the regulation of Ca^{2+}-dependent functions, including exocytosis. It has been proposed that the rate of rise of secretagogue-induced Ca^{2+} signals may be an important determinant of prolactin secretion from GH_4C_1 cells (Sato et al., 1990). The rate of rise is dose-dependent in several agonist-stimulated cell types (e.g. Johnson et al., 1999). Also, the simultaneous measurement of membrane capacitance and $[Ca^{2+}]_c$ in rat gonadotropes has revealed that fast-rising Ca^{2+} signals are most effective in activating exocytosis (Tse et al., 1997). Using *Xenopus* oocytes, Machaca and Hartzell, (1999a) compared the effects of rapid IP_3R-mediated and slower SOC-generated Ca^{2+} signals on several classes of Ca^{2+}-activated Cl^- currents. Their data indicated that the rate of rise was a determining factor in the selective activation of the currents. These observations strongly suggest the possibility that information may be coded in the rate of rise of Ca^{2+} signals.

How the rate of rise may be decoded by cells is not known at this time. By analogy to the frequency decoding of CaM kinase II, one might speculate that some enzymes possess inherent tuning to specific Ca^{2+} signal rates. On the other hand, the rate of rise could determine to what extend Ca^{2+} waves overcome cytoplasmic buffers, thereby dictating to spatial pattern of Ca^{2+} signals. Hernandez-Cruz and colleagues (1997) recently described a phenomenon in neurons where the activation of CICR depends on the rate of rise in the initial Ca^{2+} event. Since CICR represents a common mechanism for propagating Ca^{2+} signals, this finding is likely to have widespread implications.

Temporal coding of Ca^{2+} signals: Waveform

Generally, Ca^{2+} signals can be described as biphasic, monophasic and oscillatory, although many cell types show hybrid profiles. Individual cell types may generate multiple Ca^{2+} signal waveforms (e.g. Stojilkovic et al., 1992a). It is commonly thought that the biphasic Ca^{2+} signals, such as those evoked by agonists in many cell types, are related to the sequential activation of distinct signalling pathways. The Ca^{2+} signal waveform elicited by agonists is dose-dependent in many cell types (Stojilkovic et al., 1992a). The relative occurrence of various Ca^{2+} waveforms in rat gonadotropes is under endocrine and developmental control, implying that the presence of multiple Ca^{2+} signal profiles is physiologically important (Tomic et al., 1994).

Along with others, I have postulated that cells can use duration, amplitude, frequency and rate of rise coding of Ca^{2+} signals. It is unclear, at this time, whether or not cells can receive additional, more complex information from the waveform of the Ca^{2+} signals. Biphasic Ca^{2+} signals, which are common to many neuroendocrine cell types, can be thought of as a transient Ca^{2+} spike, usually

from intracellular stores, followed by a sustained plateau of extracellular Ca^{2+} influx (Catt and Stojilkovic, 1989). Since these distinct components have different origins, and since amplitude and duration can code for specific functions, it is highly likely that their combination can transduce important information. The experimental generation of Ca^{2+} elevations with more complex profiles that mimic physiological agonist-induced Ca^{2+} signals is required before these possibilities can be tested further.

Whether similar machineries are responsible for all the Ca^{2+} signal profiles in a single cell type is not known. Could the functional diversity of intracellular Ca^{2+} release channels play a role in generating these complex patterns? An answer to this question has recently been proposed by Miyakawa et al. (1999) who used B lymphoma cells that had been genetically engineered by homologous recombination to express single IP_3R subtypes or specific combinations of IP_3R subtypes. Activation of the B cell receptor evoked an immediate single spike in cells expressing only IP_3R1 and IP_3R3, while robust oscillations were seen in the IP_3R2-expressing cells (Miyakawa et al., 1999). These results indicate that the distinct properties of intracellular Ca^{2+}-release channels can lead to dramatically different Ca^{2+} signal waveforms *in situ*.

It is possible that the combination of spatial and temporal coding may also be important. Few studies have investigated this possibility in earnest. However, simultaneous and independent Ca^{2+} signals have been measured in distinct regions of single cells. For example, localized Ca^{2+} oscillations in response to low doses of agonist have been demonstrated in pancreatic acinar cells (Thorn et al., 1993). In another example, activation of purinergic receptors elicited spatially-segregated oscillations with independent frequencies in the single smooth muscle cells (Mahoney et al., 1993). Other potential modes of temporal Ca^{2+} signal coding have not been investigated experimentally. It is conceivable that information could be encoded using the delay of Ca^{2+} signals with respect to other cellular events. By analogy to the nervous system, could information be encoded in the spatio-temporal coincidence with other signalling molecules or with other Ca^{2+} signals? Perhaps the relationships between the phases of multiple independent Ca^{2+} oscillations could also encode information. These possibilities illustrate the complexity of signals that can be attained when spatial and temporal Ca^{2+} signal coding are combined. When one considers the number of Ca^{2+}-dependent cellular functions that are known to occur simultaneously, it is apparent that these interactions may be required, if not unavoidable.

Agonist-specific Ca^{2+} stores and Ca^{2+} signatures

Thus far, I have argued that the number of Ca^{2+}-dependent cellular functions necessitates a complex system for the generation of Ca^{2+} signals, and that cells do possess such capabilities. What physiological evidence substantiates this proposal? Evidence of this is beginning to emerge in the literature. For example, in human neutrophils, chemoattractant stimuli evoke Ca^{2+} release from the centrally located, IP_3-sensitive Ca^{2+} stores, whilst peripheral stores, which are IP_3-insensitive, are involved in cell adhesion (Pettit and Fay, 1998). The diversity of Ca^{2+} signals has also been examined in several other secretory cell types,

including pancreatic β-cells, pancreatic acinar cells, adrenal medullary cells and pituitary cells, as well as, cell lines derived from these tissues (see Johnson and Chang, 2000a for review).

Function- and agonist-specific Ca^{2+} signalling is also being investigated in pituitary cells. One class of pituitary cells where the specificity of Ca^{2+} signalling has been examined is the gonadotropes, which synthesizes and secrete two gonadotropic hormones, GTH-I and GTH-II (also known as follicle-stimulating hormone and luteinizing hormone, respectively). As discussed above, frequency-coded Ca^{2+} signals have been implicated in the secretion of GTH-II (Tse et al., 1993) and differential control of GTH-I and GTH-II synthesis (Haisenleder et al, 1997). It seems likely that the spatial and temporal characteristics of agonist-evoked Ca^{2+} signals may also be responsible for the differential control GTH-I and GTH-II secretion. Gonadotropes have also been a model for comparing agonist-specific Ca^{2+} signals (Stojilkovic and Catt, 1992).

Summary

All cells use similar Ca^{2+}-signalling machinery composed of conserved buffers, pumps and channels. The complexity and cell specificity is manifested in the details of how these components are arranged and activated in the generation and modulation of Ca^{2+} signals. Ca^{2+} signalling is complex, and at the same time, ingenious in its simplicity. Does Ca^{2+} signalling confer specific advantages when compared to information transduction using more structurally complicated signalling molecules? If so, is this the reason for its involvement in countless cellular functions? Although these 'why' questions cannot presently be answered, I hope to add, with work described in this thesis, to the considerable progress which has recently been made with regard to how cells accomplish agonist- and function-specific Ca^{2+} signalling.

In this chapter, I have reviewed evidence that supports the idea that multiple spatially and functional distinct Ca^{2+} stores help cells solve the potential problems associated with the simultaneous control of many targets. Clearly, the diverse properties of Ca^{2+} stores that can act independently, or together, provide the necessary functional 'richness' to create complex Ca^{2+} signals. These Ca^{2+} signals may be unique to a particular agonist or targeted to a single cellular function, or both. However, evidence that parallel, but independent, Ca^{2+} signals can simultaneously modulate various cellular functions in single intact cells remains elusive. Cultured gonadotrope and somatotrope cells of the goldfish pituitary are a suitable model system with which to examine the roles of multiple Ca^{2+} stores with regard to agonist- and function-specific Ca^{2+} signalling.

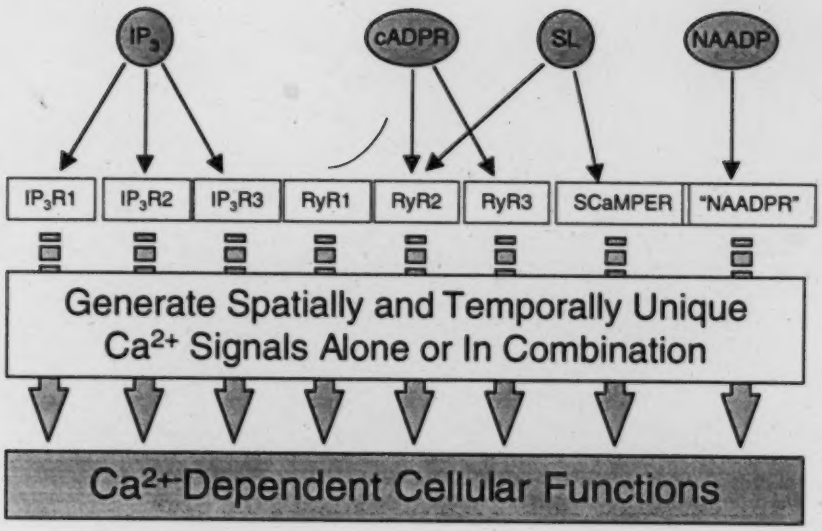

Figure 1.1. Potential functional heterogeneity of Ca^{2+} stores in neuroendocrine cells. Several Ca^{2+} release channels, and the second messenger cascades that regulate them, are shown. Each of these Ca^{2+} channel types or isoforms may generate Ca^{2+} signals with different spatial and temporal characteristics. Individual components shown also interact. Addition functional diversity that may arise from putative IP_3R and RyR heterotetramers is not shown. **Abbreviations:** IP_3, inositol 1,4,5 trisphosphate; IP_3R, IP_3 Ca^{2+}-release channel; cADPR, cyclic adenosine 5'-diphosphate ribose; RyR, ryanodine receptor Ca^{2+}-release channel; SL, Ca^{2+}-mobilizing sphingolipids; SCaMPER, sphingolipid Ca^{2+}-release-mediating protein of endoplasmic reticulum; NAADP, nicotinic acid dinucleotide phosphate; NAADPR, putative NAADP Ca^{2+}-release channel.

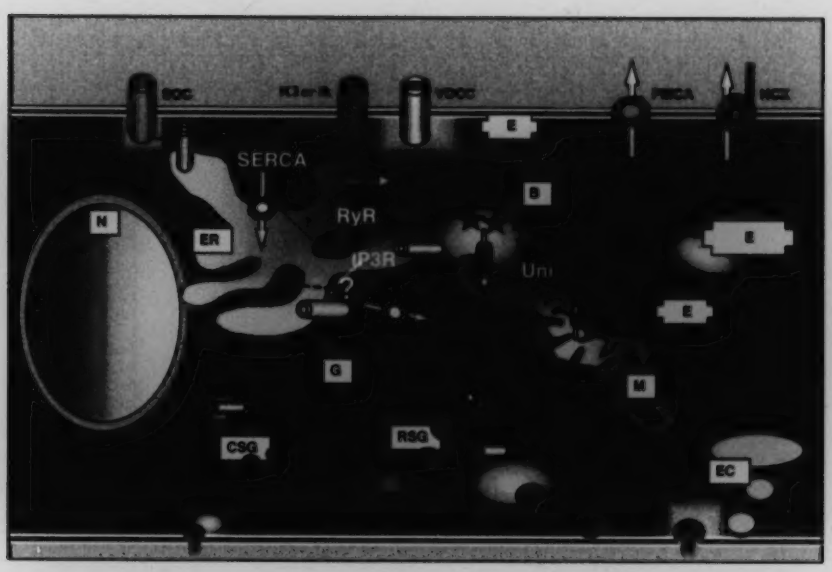

Figure 1.2. Possible roles of localized Ca^{2+} signals in neuroendocrine cells. High [Ca^{2+}] is depicted in yellow. The blue numbers correspond to the following Ca^{2+}-dependent cellular functions that may be regulated by localized Ca^{2+}signals. Local Ca^{2+} events, constricted by the presence of Ca^{2+} buffers, are recruited in the generation of global Ca^{2+} signals (1). The integration and resolution of Ca^{2+} signals coded in frequency, duration and amplitude in the nucleus can regulate the expression of specific genes (2). Localized high-Ca^{2+} environments are required for protein production and processing in the ER and Golgi (3). Ca^{2+} is required for transport and docking of secretory granules (4). Local high-amplitude Ca^{2+} domains trigger exocytosis (5) and may play a role in endocytosis (6). Since it is required for constitutive membrane fusion, localized Ca^{2+} elevations may also mediate exocytosis in the constitutive secretory pathway (7). Ca^{2+} oscillations in the mitochondria regulate metabolic enzymes (8). Numerous cytosolic and membrane-bound Ca^{2+}-activated enzymes may be preferentially controlled by local Ca^{2+} signals (9). Colocalization of VDCC/VGCC with other ion channels leads to the regulation of membrane voltage and cellular excitability (10). **Abbreviations:** E, Ca^{2+}-activated enzymes; N, nucleus; M, mitochondria; ER, endoplasmic reticulum; G, Golgi apparatus; CSG, secretory granules of the constitutive pathway(s); SG, secretory granules of the regulated pathway(s); EC, endosomal compartments; VDCC/VGCC, voltage-gated Ca^{2+} channel; SOC, store-operated Ca^{2+} channel; I_{Cl}, Ca^{2+}-activated chloride channel; I_K, Ca^{2+}-activated potassium channel; PMCA, plasma membrane Ca^{2+}-ATPase; NCX, Na^+/Ca^{2+} exchanger; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; RyR, ryanodine receptor Ca^{2+}-release channel; IP_3R, inositol 1,4,5 trisphosphate Ca^{2+}-release channel; Uni, mitochondrial Ca^{2+} uniporter. Organelles and Ca^{2+} gradients are not drawn to scale.

Chapter 2.

Agonist signal transduction in goldfish gonadotropes and somatotropes: Current models and comparison with mammalian systems

The goldfish pituitary

In goldfish, as in mammals, pituitary gland secretions regulate many important physiological functions. Anterior pituitary cells of teleost fishes receive direct innervation from neurons descending from hypothalamic nuclei (Kah et al., 1993). Thus, unlike the case in mammals, cells of the goldfish pituitary can be considered to be post-synaptic (reviewed in Ball, 1981). Two cell types, the gonadotropes and the somatotropes, offer several advantages for the study of how signals from the brain are transduced into hormone release from the pituitary. Firstly, hormone release from both cell types represents the well characterized and easy-to-measure outputs of these circuits. Studies on mixed cultures of goldfish pituitary cells allow the simultaneous monitoring of hormone secretion from both gonadotropes and somatotropes. Secondly, a reliable methodology has been validated for the identification of live gonadotropes and somatotropes in mixed culture using morphological criteria (Van Goor et al., 1994). This development was important because it has permitted experiments on single cells, without chemical pretreatments required for reverse hemolytic plaque assays. Thirdly, the signalling mechanisms mediating the secretagogue actions of several neuroendocrine regulators on both cell types have been characterized to a relatively large extent (reviewed in Chang et al., 1996; Chang et al., 2000). Furthermore, some hypothalamic factors control the activity of both cell types, thus allowing the comparison of their signalling cascades on two distinct target cell types within the same experimental preparation. The gonadotropin-releasing hormones, chicken (c)GnRH-II and salmon (s)GnRH, which stimulate both gonadotropes and somatotropes, are one such group of hypothalamic neuroendocrine regulators.

GnRH neuropeptides

GnRH is a multifunctional 10 amino acid neuropeptide that functions as a primary regulator of reproduction (King and Millar, 1995). Across all vertebrate classes, at least 14 forms of GnRH exist (King and Millar, 1995; White et al., 1997; Carolsfeld et al., 2000; JP Chang, personal communication). It appears that most, if not all, vertebrates contain at least two forms of GnRH. Although this has been known for non-mammalian vertebrates for many years, multiple GnRH forms have only recently been identified in mammals, including humans (White et al., 1997). cGnRH-II is common to many species, whereas the second GnRH is more variable (White et al., 1997). In spite of the common occurrence of two or more GnRHs within individual species, the physiological functions and signal transduction of multiple GnRHs have only been extensively characterized in goldfish, where both endogenous neuropeptides (sGnRH and cGnRH-II) have been shown to be physiological regulators of gonadotropin secretion. In addition, both sGnRH and cGnRH-II play a role in the control of growth hormone release

(reviewed in Peter and Chang, 1999). This *in vitro* dual cell/dual agonist model system has provided important insights into the hypothalamic control of pituitary function and revealed novel features of cellular signalling (Chang et al., 1996; Chang et al., 2000).

Signal transduction cascades in goldfish gonadotropes

GnRH receptors, which are members of the guanine nucleotide-binding (G)-protein-coupled receptor superfamily, have been cloned and localized to the goldfish pituitary (Illing et al., 1999; Leung and Peng, 1996). The signal transduction of both GnRHs requires PKC, which is probably activated by PLC-mediated DAG production (Illing et al., 1999; Chang et al., 1996). It is thought that PKC regulates Ca^{2+} entry through L-type VGCC (Chang et al., 2000). Interestingly, calmodulin appears to mediate the long-term (2 hours) regulation of GTH-II release by GnRH, but is not involved in acute GnRH-signalling (Jobin et al., 1996). GnRH-stimulated GTH-II release is also sensitive to extracellular Na^+ removal, although this effect is not due to a requirement for TTX-sensitive voltage-gated Na^+ channels (Van Goor et al., 1996). Apamin-sensitive Ca^{2+}-activated K^+ channels are also not involved in GnRH action (Chang et al., 2000).

The control of GTH-II secretion involves agonist-specific second messenger pathways in gonadotropes (discussed in depth in Chapter 4). For example, GTH-II release evoked by sGnRH, but not cGnRH-II, involves arachidonic acid (AA) signalling (Chang et al., 1995). Importantly, sGnRH-stimulated GTH-II is significantly less sensitive to manipulations that reduce the availability of extracellular Ca^{2+} (Jobin and Chang, 1992; Chang et al., 1995). This apparent involvement of intracellular Ca^{2+} stores is correlated with the observation that only sGnRH generates significant levels of IP_3 from mixed cultures of goldfish pituitary cells (Chang et al., 1995).

Although treatments that elevate cAMP levels stimulate GTH-II release, PKA activation is not involved in GnRH-stimulated hormone release from goldfish gonadotropes (Chang et al., 1992). Instead, GTH-II release evoked by pituitary adenylate cyclase activating polypeptide (PACAP) requires the cAMP/PKA pathway (reviewed in Chang et al., 2000; JP Chang, NR Wirachowsky, P Kwong, JD Johnson, manuscript submitted), which is relatively insensitive to manipulations of extracellular Ca^{2+} (Chang et al., 1992). In addition to sGnRH, cGnRH-II and PACAP, GTH-II release may also be controlled, at the level of the goldfish pituitary, by a plethora of other neuroendocrine regulators, including norepinephrine, serotonin, neuropeptide Y and activin/inhibin (Chang et al., 2000). However, comprehensive studies into the signalling cascades of these other neuroendocrine regulators have not been reported. The major inhibitory influence on basal and agonist-evoked GTH-II release is supplied by dopamine through D2-like receptors, which have been shown to couple negatively to VGCC (reviewed in Chang et al., 2000).

Signal transduction cascades in goldfish somatotropes

As is the case in goldfish gonadotropes, GnRH signalling requires PKC activation in somatotropes (Chang et al., 1996). Pretreatment in conditions that

reduced the availability of extracellular Ca^{2+} inhibits sGnRH- and cGnRH-II-stimulated GH release (Wong et al., 1994). Calmodulin also is involved in the acute control of GH release by GnRH (Chang et al., 1996). In addition, a dependence on extracellular Na^+ and Na^+/H^+ exchange has also been described (reviewed in Chang et al., 2000). So far, no differences between the signal transduction of sGnRH and cGnRH-II in somatotropes have been reported. Neither cAMP nor AA signalling cascades are involved in GnRH-stimulated GH release, though both second messenger systems can positively couple to GH secretion (Wong et al., 1994). In contrast, both dopamine and PACAP evoke GH release though signal transduction cascades which involve cAMP and VGCC (Wong et al., 1994; reviewed in Chang et al., 2000; NR Wirachowsky, P Kwong, WK Yunker, JD Johnson, JP Chang, manuscript submitted). Other than the two GnRHs and PACAP, dopamine (via D1 receptors), GH-releasing hormone and neuropeptide Y can release GH from goldfish somatotropes. GH release is inhibited by somatostatin, norepinephrine and serotonin (reviewed in Chang et al., 2000).

Signal transduction cascades in mammalian gonadotropes and somatotropes

Mammalian (m)GnRH mechanisms of action have been extensively studied in mammalian gonadotropes (Hille et al., 1995; Stojilkovic and Catt, 1995). mGnRH signalling begins with the activation of a $G_{q/11}$-protein leading to PLC-mediated generation of IP_3 and DAG (Noar et al., 1998). Although extracellular Ca^{2+} is required for the maintenance of prolonged Ca^{2+} signalling (>10 minutes), acute mGnRH control of GTH exocytosis is mediated entirely by intracellular Ca^{2+} stores (Tse et al., 1993; Stojilkovic et al., 1994; Tse et al., 1997; Chang et al., 1988). This Ca^{2+} pool, which mediates frequency-modulated $[Ca^{2+}]_c$ oscillations, is sensitive to both IP_3 and SERCA pump inhibitors (Tse et al., 1993; McArdle and Poch, 1992). The oscillatory activity of the Ca^{2+} stores is linked through the modulation of Ca^{2+}-sensitive K^+ channel activity to oscillations in membrane excitability which activate VGCC and permit the refilling of the stores (Stojilkovic et al., 1992b; Stojilkovic et al., 1993; Van Goor et al., 1999). Thus, an IP_3-sensitive Ca^{2+} pool is necessary and sufficient for the regulation of hormone release from rat gonadotropes. Although mRNA for the RyR has been isolated from the rat pituitary (Sundaresan et al., 1997), a role for Ry-sensitive Ca^{2+} stores in gonadotrope function remains equivocal. Other second messengers have been implicated in GnRH signalling, including AA and PKC (Naor et al., 1998). For example, PKC interacts with Ca^{2+}-dependent signalling cascades by sensitizing the secretory apparatus (Jobin et al., 1995). In addition to PKC, long-term GnRH signalling involves mitogen-activated protein kinase cascades (Noar et al., 1998). Various cell lines, including αT-3 cells, have been used in parallel studies. However, spurious results in regard to stimulation-secretion coupling have been reported (Trueta et al., 1999).

The principle stimulatory neuroendocrine regulators of growth hormone (GH) release from rat somatotropes is growth hormone-releasing hormone (GHRH; Frohman et al., 1992; Chen et al., 1994; Petersenn and Schulte, 2000). Occupation of the G-protein-coupled GHRH receptor leads to the acute activation

of adenylate cyclase, the cAMP/PKA cascade and Ca^{2+} influx (Frohman and Jansson, 1986; Lussier et al., 1991; Frohman et al., 1992). It has been proposed that the control of membrane potential, and therefore VGCC-mediated Ca^{2+} flux, is the major determinant of acute GH release (Chen et al., 1994). Like many agonists, GHRH generates Ca^{2+} signals which are biphasic, having a rapid 'peak' followed by a sustained 'plateau'. Among G-protein-mediated signalling cascades in non-neuronal cells, this is one of the only examples where the transient phase has been attributed to extracellular Ca^{2+} influx, while the sustained phase is apparently due to the release from stores (Chen et al., 1994; see also Frohman et al., 1992). A synthetic compound, growth hormone-release hexapeptide, stimulates GH release through cAMP-independent pathways involving PLC, IP_3 and DAG (Frohman et al., 1992; Chen et al., 1994). Although Ry-sensitive Ca^{2+} stores participate in the Ca^{2+} signals evoked by GHRH and GHRP (Petit et al., 1999; Herrington and Hille, 1994), no evidence has been reported that RyR is involved in GH release. Ca^{2+} signalling has also been investigated in GH-related cell lines, such as GH_3 and GH_4C_1 cells, which may not be similar to somatotropes in all manners.

General goals and approaches

The principal aim of this thesis is to explore the possibility that multiple intracellular Ca^{2+} stores participate in physiologically relevant processes in gonadotropes and somatotropes of the goldfish pituitary and to test whether this involvement is agonist-specific and/or function-specific. Since the agonist specificity of signal transduction is well established in gonadotropes (reviewed in Chang et al., 2000), they are an excellent model system for such studies. Goldfish somatotropes are also under multifactorial regulation and may, therefore, also exhibit agonist-specific Ca^{2+}-dependent signal transduction. Almost nothing is known about the nature of intracellular Ca^{2+} stores, or their role in agonist signalling, in goldfish pituitary cells. As a partial solution to this void in our understanding of pituitary physiology, this thesis represents a broad pharmacological survey of the roles of multiple intracellular Ca^{2+} stores in goldfish gonadotropes and somatotropes, rather than a more detailed characterization of a single Ca^{2+} store. I believe that such an approach will provide the greatest benefit to future studies by acquiring a large amount of information regarding the complexity and multiplicity of intracellular Ca^{2+} stores in these two cell types.

The relative roles of various intracellular Ca^{2+} stores will be evaluated, primarily, in the context of a well established physiological endpoint, hormone release. For the purposes of this thesis, the term "intracellular Ca^{2+} store" will be given a rather abstract definition. Aside from the obvious criterion as an intracellular Ca^{2+} storage compartment, Ca^{2+} stores can be defined based on their function in a cell. For example, two intracellular Ca^{2+} stores can be considered distinct entities if they are differentially involved in specific signalling cascades controlling agonist-stimulated Ca^{2+} signalling or hormone release. This involvement can be revealed using pharmacological tools known to affect the function of specific Ca^{2+} handling proteins. The functional definition of

intracellular Ca^{2+} stores as a signalling component does not necessarily permit the identification of the ultrastructural location, such as an organelle or part of an organelle, or the exact molecular characterization of the Ca^{2+}-release channels and pumps that regulate the ion fluxes. In this regard, the extent to which pharmacology can offer insight is variable.

Therefore, it is appropriate to discuss some strengths and limitations of the pharmacological approach to the study of signal transduction. Although the tools of molecular biology offer exquisite specificity, pharmacological tools offer the ability to change the functioning of cells in a rapid and reversible manner and to tightly control the degree of modulation. In addition, such manipulations can be performed on non-transformed cells. However, all pharmacological studies require assumptions regarding specificity, which can be very difficult to ensure, especially in systems in which the drug has not been fully characterized. Notwithstanding, with proper controls and cautious interpretation of the data, I believe the pharmacological approach is best suited to the type of studies presented in this thesis. Herein, I endeavor to test for the involvement of as many pharmacologically distinguishable Ca^{2+} stores as possible.

Generally, drugs will be employed at the maximal dose that can be considered, based on the available literature, to be specific. In most cases, this literature is based on experiments conducted on mammalian cells, or even cell lines. Thus, future studies may require internal validation of the specificity of the drugs used in this thesis. Nonetheless, the simultaneous monitoring of both gonadotrope and somatotrope responses also offers an additional level of control in the event that treatment effect differ between the two cell types. Experiments designed to elucidate the signal transduction of both GnRHs will be conducted using 100 nM GnRH, which is a maximal dose for both sGnRH and cGnRH-II in previous hormone release studies (Chang et al., 1990). It is important to state that the signal transduction cascades evoked by different doses of GnRH, and agonists in general, may not be identical. Currently, there is no data regarding the possible differences in the signal transduction of different doses of GnRH in goldfish pituitary cells, although such studies would be extremely valuable.

Previous studies have hinted that aspects of Ca^{2+}-dependent signal transduction in goldfish pituitary cells may vary with the reproductive status of the donor fish (e.g. Chang et al., 1995, reviewed in Chang et al., 2000). However, no systematic studies of such seasonal differences have been reported. Likewise, a full seasonal study of the involvement of various Ca^{2+} stores is beyond the scope of this thesis. Experiments will be conducted at all times during the seasonal reproductive cycle. Generally, data will be pooled from all similar experiments, irrespective of the time of year when they were conducted. When the data cannot be pooled, due to significant difference between the results of experiments conducted during different sexual stages, they will be considered separately.

Specific goals

The general goal of this thesis, to investigate the complexity and multiplicity of intracellular Ca^{2+} stores in pituitary cells, can be divided into several specific experimental studies. These specific hypothesis-based studies are

aimed at resolving many new and previously unanswered questions pertaining to Ca^{2+}-dependent signal transduction in goldfish gonadotropes and somatotropes.

For example, since previous studies have demonstrated a degree of agonist-specificity in the signal transduction of sGnRH and cGnRH-II, Chapter 4 examines whether these closely related endogenous neuropeptides generated dissimilar Ca^{2+} signals. A quantitative method for comparing Ca^{2+} signals is also presented. The goal of Chapter 5 is to test the hypothesis that intracellular Ca^{2+} stores play a role in GnRH signalling leading to GTH-II release and to investigate whether the involvement of specific Ca^{2+} stores differs between the two GnRHs. One such agonist-specific intracellular Ca^{2+} signalling component, caffeine-sensitive Ca^{2+} stores, are involved in GTH-II release evoked by sGnRH, but not cGnRH-II. Results in Chapter 6 provide a partial characterization of the signal transduction mediating caffeine-stimulated to GTH-II release.

As was the case in gonadotropes, previous studies of the extracellular Ca^{2+} dependence of GnRH-stimulated GH release were inconclusive because inhibitors of extracellular Ca^{2+} entry were applied to the cells for periods long enough that intracellular Ca^{2+} stores could have been depleted. The goal of Chapter 7 is to establish the role of intracellular Ca^{2+} stores in GH release stimulated by sGnRH and cGnRH-II. Results in Chapters 5, 6 and 7 provided evidence for a dominant role for intracellular Ca^{2+} stores in acute GnRH-stimulated hormone release in both cell types. Moreover, these findings suggested that the Ca^{2+}-dependent signal transduction of sGnRH and cGnRH-II in both cell types displayed an unusual pharmacological profile. Chapter 8 compares the role of Ry-sensitive Ca^{2+} stores, which are not normally considered to be an agonist-sensitive Ca^{2+} store in most cell types, between both peptides in both gonadotropes and somatotropes.

Since recent data have implicated mitochondria in the regulation of Ca^{2+} signalling and agonist-evoked hormone release in several cell types (Duchen, 1999), the goal of Chapter 9 is to examine the role of mitochondrial Ca^{2+} buffering in goldfish somatotropes. Furthermore, I sought to determine whether mitochondrial Ca^{2+} handling interacts with other Ca^{2+} stores in an agonist-specific manner.

As an extension of ideas presented in Chapter 4, experiments described in Chapter 10 test the hypothesis that the rate of rise, and not the amplitude, of Ca^{2+} signals plays an important role in the control of hormone release from gonadotropes. In the process, it is revealed that a certain class of intracellular Ca^{2+} store appears to be uncoupled from the stimulatory control of exocytosis. This leads to the novel hypothesis that Ca^{2+} stores may be endowed with function specificity. The last two data chapters deal with this possibility by comparing the effects of modulating two pharmacologically distinct Ca^{2+} stores on several Ca^{2+}-modulated elements of the extended secretory pathway. Specifically, the role of two classes intracellular Ca^{2+} stores in hormone secretion, storage, production and gene transcription are compared in gonadotropes (Chapter 11) and somatotropes (Chapter 12). The findings of this thesis are synthesized (Chapter 13) and used to argue that cells utilize multiple intracellular Ca^{2+} stores to encode information with agonist and function specificity.

Chapter 3.

Measuring multiple cellular functions and quantification of spatial and temporal Ca^{2+} signal characteristics in goldfish pituitary cells

Reagents

Stocks of biochemically synthesized sGnRH ([Trp^7, Leu^8]GnRH), cGnRH-II ([His^5, Trp^7, Tyr^8]GnRH) and pituitary adenylate cyclase-activation polypeptide (PACAP; mammalian $PACAP_{38}$) were purchased from Peninsula Laboratories (Belmont, CA, USA) and were made up in distilled deionized water. Thapsigargin (Tg) and (-)-BayK 8644 (Calbiochem, LaJolla, CA) were dissolved in ethanol. High purity ryanodine (99.5%), 2-bis-(2-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), 8-(N,N-diethyl-amino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8), cyclopiazonic acid (CPA), 2,5-di-(*t*-butyl)-1,4-hydroquinone (BHQ), xestospongin C, brefeldin A, okadaic acid, ionomycin (Calbiochem, LaJolla, CA), 12-O-tetradecanoylphorbol 13-acetate (TPA), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), forskolin (Sigma, St. Louis, MO), dantrolene and ruthenium red (RBI, Natick, MA) were dissolved in dimethyl sulphoxide. At the concentrations used in these studies, DMSO and ethanol do not affect $[Ca^{2+}]_c$, ionic currents or hormone release (final concentration < 0.1 %; data not shown). Caffeine (RBI, Natick, MD) and cyclohexamide (ICN, Aurora, OH) were dissolved directly into testing media.

Depolarizing media (30 mM KCl) was made by equimolar substitution of NaCl with KCl. Nominally Ca^{2+}-free solutions were made by omitting all calcium salts and replacing them with equimolar NaCl. EGTA was not added since completely Ca^{2+}-free conditions may establish a driving force that favors the loss of Ca^{2+} from intracellular stores. The 'Ca^{2+}-free' media used in the present study would, at the very least, dramatically reduce the driving force of extracellular Ca^{2+} entry.

Animals

All animal use protocols were approved by the University of Alberta Department of Biological Sciences Animal Care Committee in accordance with national guidelines. Male and female goldfish from Aquatic Imports (Calgary, Canada) were maintained in flow-through aquaria (1800 L) at 16-20 °C as described previously (Chang et al., 1990). The photoperiod in the aquatic facility is adjusted weekly according to the local sunrise and sunset times (Edmonton, AB, Canada). Under controlled conditions, the reproductive stage of goldfish maintained in the laboratory can be accurately estimated by the time of year (Habibi et al., 1989; Lo and Chang, 1998). For a large part of the year (late May-September) goldfish are in a state of gonadal regression (gonadosomatic index (GSI) <2%). Over the winter, their gonads progress from early recrudescence (Dec-January) to late recrudescence (February-March). Full sexual maturation is achieved in March and April (GSI >6%). Moreover, the sexual stage of the donor

fish can be easily recognized in our cell column perifusion experiments. The basal and GnRH-stimulated release of both GTH-II and GH *in vitro* display distinct features depending on the reproductive status of the donor fish (Lo and Chang, 1998; see Chapter 8). Unless otherwise stated, results from experiments that spanned several sexual stages were similar and therefore pooled.

Pituitary cell culture

Pituitaries from male and female goldfish, were dispersed using a trypsin/DNAase protocol that has been described previously (Chang et al., 1990). Dispersed cells were cultured overnight on poly-L-lysine-coated coverslips (0.25 million cells/coverslip, 1 ml media) for imaging experiments (Johnson et al., 1999), preswollen Cytodex beads (1.5 million/dish, 6 ml media) for cell column perifusion studies (Chang et al., 1990), Primaria surface-modified tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) for static incubation experiments designed to measure long-term hormone secretion/contents (24 well plates; 0.25 million cells/well; 1 ml media) or mRNA levels (6 well plates; 2 million cells/well; 4 ml media) in Medium 199 with Earle's salts (Gibco, Grand Island, NY) supplemented with 2.2 g/l $NaHCO_3$, 25 mM HEPES, 100,000 U/l penicillin, 100 mg/l streptomycin, and 1% horse serum (pH adjusted to 7.2 with NaOH) at 28°C, 5% CO_2, and in saturated humidity (Chang et al., 1990).

Cell identification

Goldfish gonadotropes and somatotropes were identified under Nomarski differential interference contrast optics using a specific set of morphological criteria that have been empirically validated (Van Goor et al., 1994). Briefly, gonadotropes (cell diameter 12-19 μm) contain a small kidney-shaped nucleus (with a prominent nucleolus) located against one side of the cell. Large globules and irregular inclusions are also clearly visible. Cells that exhibit all of these morphological characteristics are distinct from other cell types in the mixed population. Indeed, we have previously shown that the identification is ~95% accurate for predicting cells that stain positive for GTH-II (Van Goor et al., 1994). Similarly, >95% of morphologically identified cells respond to GnRH with Ca^{2+} signals (Chapter 4; Johnson et al., 1999). We have estimated that 50% of gonadotropes are represented in the identifiable subpopulation (Johnson et al., 1999). Somatotropes are spherical (cell diameter 9-11 μm) with a large ovoid nucleus, which occupies ~50% of the image plane and is located eccentrically. Somatotropes are also characterized by a prominent nucleolus (located at the center of the nucleus) and numerous granules and cytoplasmic extensions (identification accuracy >95%; Van Goor et al., 1994). The use of morphological identification eliminates the need for pretreatment with exogenous and potentially bioactive materials such as those required for hemolytic plaque assays.

Ca^{2+} imaging

Stock solutions of 10 mM Fura-2 AM were made up in DMSO with 20% (v/v) Pluronic F-127 and sonicated for 10 minutes. Cells were stably and evenly loaded with Fura-2 delivered by incubation with 10 μM Fura-2 AM (Molecular

Probes, Eugene, OR.) for 40 minutes at 28° C, after which cells were washed three times. These loading conditions have been optimized for our cell types, which seem to take up AM-esters less efficiently than mammalian cells. Fura-2 can only be considered a useful index of $[Ca^{2+}]_c$ if loading conditions have been optimized to reduce dye compartmentalization within organelles. Even then, we can only assume that de-esterified Fura-2 is predominantly restricted to the cytosol and that the contribution of lumenal Fura-2 fluorescence, which would likely be saturated, is minimal. In addition to the reality of dye compartmentalization, other limitations of Fura-2 imaging include: 1) incomplete characterization of the cellular milieu, especially with respect to pH, other ion fluxes and viscosity, 2) the possibility that some drugs may autofluoresce or quench the Fura-2 signal, and 3) variable and incomplete loading and de-esterification. Although such factors may affect the absolute value of the $[Ca^{2+}]_c$ estimates, the conclusions drawn in this thesis are based primarily on relative $[Ca^{2+}]_c$ changes between various treatments.

Loading, washes and experiments were conducted in imaging media consisting of Medium 199 with Hank's salts (Gibco or NIH media unit, Bethesda, MD, USA, gift from Dr. S. S. Stojilkovic; phenol red was omitted for imaging experiments) supplemented with 2.2 g/l $NaHCO_3$, 25 mM HEPES, 100,000 U/l penicillin, 100 mg/l streptomycin, 0.1% BSA; pH adjusted to 7.2 with NaOH. Media were applied using a pump-driven perifusion system at a rate of 1 ml/min, at ~21°C. The bath volume was less than 100 µl and the inflow placed close to the cell of interest; complete solution changes were achieved in the vicinity of the cell ~10 seconds.

Fura-2, in cells which had been selected using the morphological criteria, was excited with a Hg-Xe arc lamp (Hamamatsu, Japan) at 340 nm and 380 nm wavelengths using a computer controlled filter wheel (Empix Imaging, Mississauga, ON). Emission fluorescence, at 510 nm, was recorded through the 100x oil-immersion objective (1.3 N.A. Fluor) of a Zeiss inverted microscope (Carl Zeiss Canada, Don Mills, ON) using a Paultek Imaging ICCD video camera (Grass Valley, CA) and a frame-grabber board on a Macintosh Quadra computer running custom software (gift from S. B. Kater, University of Utah School of Medicine). Camera integration was not used. Camera gain was sometimes used to ensure a high signal to noise ratio. Neutral density filters (Omega Optical, Brattleboro, VT) were employed to ensure that F_{340} and F_{380} measurements were within the sensitivity range of the camera and to prevent photo-bleaching. Due to the long duration of the experiments, the image capture frequency did not exceed 10 seconds, in order to minimize photobleaching.

Spatial and temporal quantification of Ca^{2+} signals

Ca^{2+} signals were analyzed off-line using a Macintosh G3 computer using the public domain program NIH Image (version 1.61, written by Wayne Rasband at the U.S. National Institutes of Health and available on the Internet by anonymous FTP from zippy.nimh.nih.gov) and Ca^{2+}Ratiometrics (version 1.3, a user-contributed macro custom-written by CJH Wong, Division of Neuroscience, University of Alberta). This macro automatically subtracts background fluorescence and thresholds the image (Silver *et al.*, 1992). F_{340} and F_{380} ratios

Grynkiewicz and co-workers (Grynkiewicz *et al.*, 1985). R_{max} (3.85), R_{min} (0.76) and K_d (465.9) values were determined empirically in triplicate using an *in vitro* calibration kit (Molecular Probes, Eugene, OR.). Ca^{2+} estimations are plotted on a linear scale; our measurements fall in the 'linear' part of the sigmoidal ratio vs Ca^{2+} standard curve.

Temporal analysis was carried out as previously described (Johnson et al., 1999). The 5-minute drug application periods were divided into 1-minute bins. Ca^{2+} responses were deemed significant if the mean $[Ca^{2+}]_c$ measurement for any bin during the drug application exceeded the mean of the pre-application bin + 1 SEM. According to these criteria, basal $[Ca^{2+}]_c$ of goldfish gonadotropes does not vary (Johnson et al., 1999; Chapter 4). Whole-cell latency of the response was determined as the time from drug application to the first data point that was 1 SEM above the pre-application bin. It is important to distinguish this parameter from the actual time that the Ca^{2+} signal began since Ca^{2+} signals could usually be detected in some area of the cell by the first time-point after the drug application. Therefore, whole-cell latency likely reflects the time required to generate a large, measurable Ca^{2+} event from elementary Ca^{2+} signalling units. The maximum amplitude was the highest average cytoplasmic Ca^{2+} measurement taken during the application of the drug minus the pretreatment baseline value. This parameter is also merely an estimate, due to uncertainties related to the calibration of Fura-2 and the fact that global estimations of $[Ca^{2+}]_i$ misrepresent regions of higher localized $[Ca^{2+}]_i$. The slope to peak amplitude from the time of the drug application was calculated as an estimate of the rate of rise to maximum amplitude. Similar results were obtained if the rising phase of the Ca^{2+} signal was fitted using linear regression. The net rate of rise of the global Ca^{2+} signal approximated in the present study is obviously not a measurement of the actual rate of release from Ca^{2+} channels. Nonetheless, we and others (Tse et al., 1997) have suggested that the former is at least proportional to the latter. The average increase in $[Ca^{2+}]_c$ above baseline was calculated over the duration of the Ca^{2+} signal. In cases where data from experiments were pooled, Ca^{2+} measurements were normalized to the resting Ca^{2+} immediately prior to the treatment period (average of 4 measurements; referred to as % pretreatment). It is important to note that Ca^{2+} signal latency values were overestimated because one sample period (15 seconds) was added to the latency measurement of all responses to reflect the typical temporal resolution in this study. An additional one-half of the sample period (8 seconds) was also arbitrarily added to each 'time to maximal amplitude' value to prevent infinite rate of rise values where the first significant point (latency) was also the maximum amplitude. Representing Ca^{2+} determinations as absolute values or as normalized values did not affect the conclusions reaching in this thesis.

Large scale Ca^{2+} gradients across the cell image were quantified as follows. Ca^{2+} levels in four approximately equal quadrants of the cell were measured for all images of a given experiment using Ca^{2+}Ratiometrics. Quandrants were selected manually, with the intersection being approximately in the center of the cell (as in Fig. 3.1C). We reasoned that the division of cells into quadrants was all that was warranted by the spatial resolution of our images, and

that such a strategy would be conservative by uncovering robust gradients only. Vectors were calculated as follows. Ca^{2+} values of diagonal quadrants were subtracted from each other to give two orthogonal vectors (i.e. 1-3 and 2-4 in Fig. 3.1C). The resultant of these two vectors yielded the direction and magnitude of the instantaneous macroscopic Ca^{2+} gradient. The angle of this vector was subsequently corrected for the nucleus (made to be North = 0°) by subtracting the angle of a line, orginating at the center of the cell and bisecting the nucleous (which is in the center of the gonadotrope nucleus), from the vertical.

To generate graphs, vectors were expressed on a series of polar coordinates versus time. Each graph contains a scale arrow which depicts a gradient in the nuclear direction (higher in area around nucleus) for comparison with the vectors from the experiments. In some graphs, the direction of all the vectors, as well as this scale arrow, were arbitrarily rotated 90° to better reveal the changes in vectors over time. Groups of vectors were compared by averaging the amplitude and the direction values, of the 'time-averaged' vectors, separately. 'Time averaging' involved the separate averaging of both components of multiple vectors over a period of time in a single cell.

In addition to developing and migrating cells (Silver, 1996; Hahn et al., 1992; Brundage et al., 1991; Yumura et al., 1996), and strongly polarized sensory and exocrine cell types (Restrepo et al., 1993; Lischika and Schild, 1993; Tinel et al., 1999; reviewed in Petersen 1999), Ca^{2+} gradients have been measured in glucose-stimulated pancreatic β-cells (Martin et al., 1997) and adrenal chromaffin cells during muscarinic stimulation (Cheek et al., 1989). We used the vector analysis approach to show that goldfish gonadotropes and somatotropes maintain robust and long-lasting Ca^{2+} gradients, even in the absence of stimulation (Fig. 3.1). These gradients were dynamic, Ca^{2+}-dependent (Fig. 3.2), and were differentially modulated by 2 μM Tg and 100 μM TMB-8 (Fig. 3.3, 3.4) which antagonize the loading and mobilization of intracellular Ca^{2+} stores, respectively. The magnitude of the spatial Ca^{2+} gradients is likely underestimated because Fura-2, a mobile Ca^{2+} buffer, has the effect of dissipating gradients. Collectively, our data demonstrate quantitatively, for the first time in an endocrine cell type, that Ca^{2+} gradients can be maintained, in absence of agonist-stimulation, and that these gradients may be subject to modulation by treatments that affect Ca^{2+} mobilization from a variety of sources. Although these measurements clearly do not faithfully represent the underlying mechanisms of Ca^{2+} gradient generation, the fact that unique and dynamic Ca^{2+} patterns are formed provides at least some insight into the spatial and temporal complexity of Ca^{2+} signalling and 'resting' Ca^{2+} homeostasis.

Measurement of hormone release, storage and production

For dynamic measurements of acute hormone release, dispersed pituitary cells (on Cytodex beads) were perifused at 18°C with testing medium as described previously (Chang et al., 1990). Media was collected in 30-second, 1-minute or 5-minute fractions and stored at -26°C. Hormone release was also measured over 2 hours using a static incubation protocol in testing medium under the culture conditions described above (0.25 million cells/well; Chang et al., 1990).

Prolonged cellular hormone contents and hormone secretion were assayed in testing medium under static incubation conditions (Chang et al., 1990). In experiments where cell contents were assessed, cells were lysed following the removal of testing medium by the addition of distilled deionized water, sonication, and repeated freeze/thaw cycles. Samples were stored at -26°C prior to radioimmunoassay. Prolonged exposures to the treatments used in this study did not reduce the cell number or the viability of the pituitary cell cultures as assessed by Trypan Blue exclusion; total RNA extracted from the cultures also did not vary.

Radioimmunoassay and data analysis

Hormone content of samples from both cell column perifusion and static incubation experiments were determined by separate, validated RIAs for GTH-II (Van der Kraak et al., 1992; Peter et al., 1984) and GH (Marchant et al., 1987). For studies of hormone secretion in perifusion conditions, values are normalized to the mean of the first five measurements (% pretreatment). Net hormone release responses of perifused cell columns to GnRH were quantified by integrating the area under the curve for the 20 minutes after the application of GnRH (except where indicated in the Figure legends). Each time point was subtracted from the pre-pulse mean, defined as the average of the three time points before the GnRH challenge. Data from static incubation experiments were normalized to untreated controls (expressed as % control).

Measurement of hormone gene expression

RNA extraction and Northern blot analysis of dispersed goldfish pituitary cells were optimized from protocols designed for pituitary fragments (Huggard et al., 1996a, Huggard et al., 1996b). Briefly, total RNA was isolated with Trizol Reagent (Gibco, Grand Island, NY) followed by the phenol-chloroform method (Chomczyski and Sacchi, 1987). RNA was quantified and checked for purity using a spectrophotometer (A_{260}/A_{280}). Treatments did not affect the amount of RNA isolated from individual wells. RNA (5 µg/well) was loaded and resolved on a 1.2% agarose/formaldehyde gel before being transferred to a Hybond N^+ membrane (Amersham, Arlington Heights, IL) using capillary action in the presence of 20 × SSPE (1 × SSPE (in M) = 0.3 NaCl, 0.02 $NaH_2PO_4 \cdot H_2O$, 0.002 EDTA, pH 7.4). Membranes were prehybridized for 1 hour and hybridized for 2 hours at 60°C with a specific cDNA probes for goldfish GH or GTH-II β subunit (Huggard et al., 1996a, Huggard et al., 1996b; labeled with [α-^{32}P]-deoxycytidine 5'-triphosphate using the random primer method, 3000 Ci/mM, Amersham) in Rapid Hybridization Buffer (Amersham). Membranes were then subjected to a series of SSPE washes (with 0.1% SDS) of increasing stringency (up to 0.1 × SSPE). Membranes were then exposed to film. Although ethidium bromide-stained gels were imaged to ensure equal loading of lanes, membranes were also stripped and rehybridized with a probe for 18s ribosomal RNA that served as an internal standard. Treatments caused no observable change in 18s levels. Autoradiograms were scanned and quantified using the densitometry function of

NIH Image 1.62 (NIH, Bethesda, MD). GH or GTH-II β subunit mRNA values were normalized to the internal control (18s levels) for each lane.

Statistical analysis

Data were analyzed using Student's unpaired *t*-test or one-way ANOVA (followed by Fisher's protected least significant difference post-hoc test). Mann-Whitney U test was used where non-parametric tests were appropriate. Where indicated, paired data were analyzed using paired *t*-test, or Wilcoxon signed-rank test when non-parametric tests were appropriate. Differences were considered to be significant when $P < 0.05$. Results are presented as mean ± SEM.

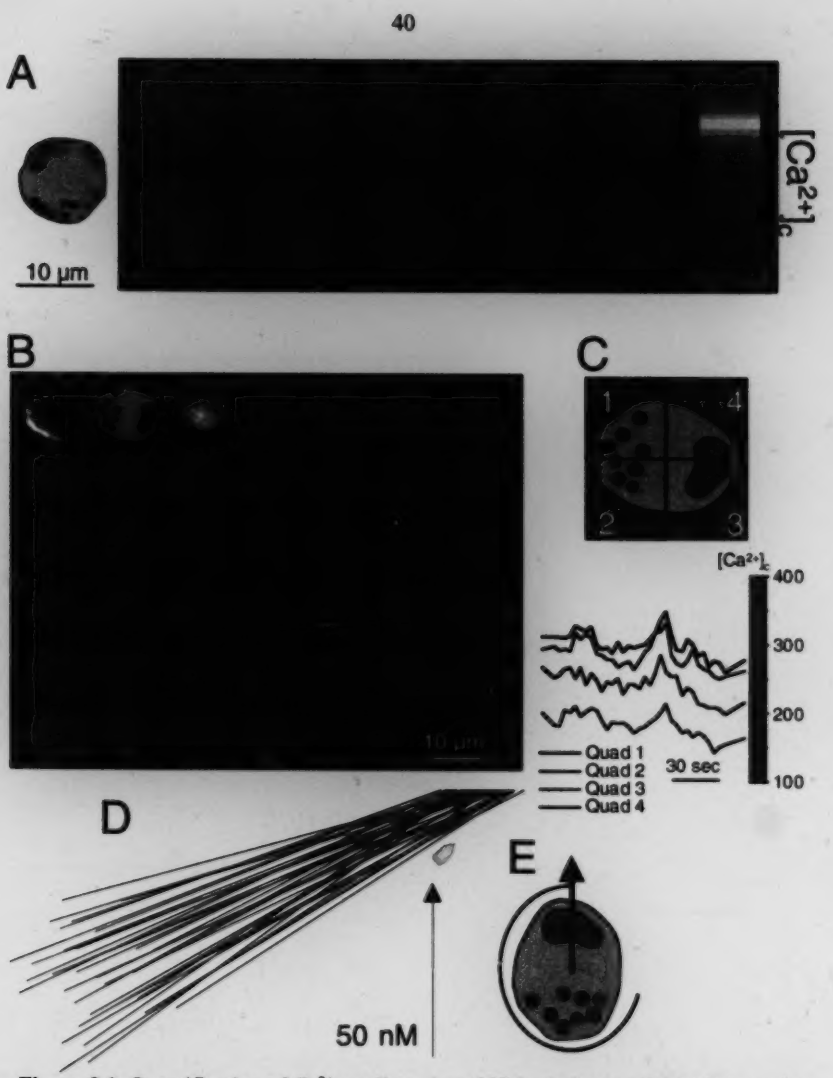

Figure 3.1. Quantification of Ca^{2+} gradients in goldfish pituitary cells. A) False colour images of spatial heterogeneities in $[Ca^{2+}]_c$ in an unperifused identified gonadotrope. Images were collected every 10 seconds. *Inset* shows a map of cellular structures visible under DIC optics. B) $[Ca^{2+}]_c$ gradients between the nuclear region and the non-nuclear regions can persist over time. Images were recorded every 4 seconds. Gradients do not result from dye loading heterogeneities. C) Recording of Ca^{2+} levels in quadrants (*Inset*) from same cell as in B. D) $[Ca^{2+}]_c$ gradient amplitude and direction were calculated from Ca^{2+} determinations for each quadrant and expressed as a series of polar plots (data are from the same cell as C). E) The angles of the vectors were corrected for the position of the nucleus (a line bisecting the nucleus was set as 0°.

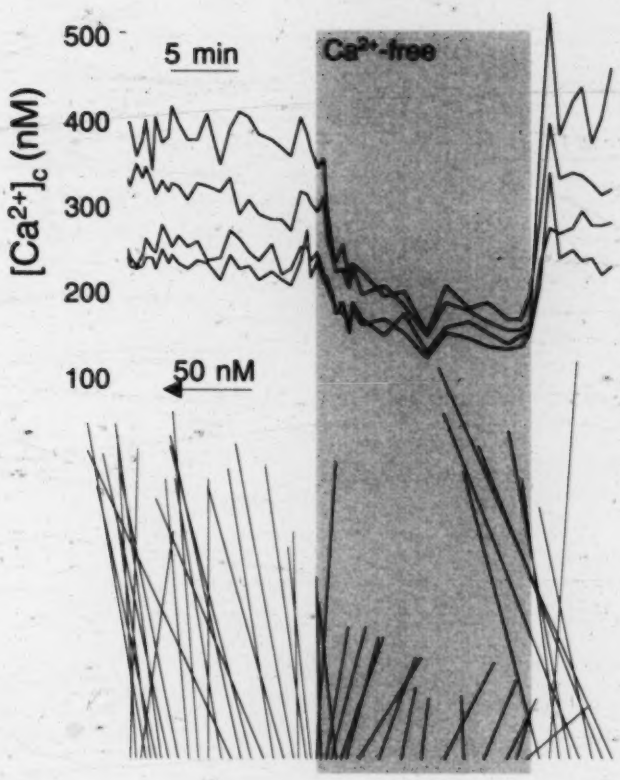

Figure 3.2. Modulation of basal Ca^{2+} gradients by extracellular $[Ca^{2+}]$. Spatial $[Ca^{2+}]_c$ levels in an identified gonadotrope are expressed at quadrant traces and vectors before, during and after exposure to nominally Ca^{2+} free media (*gray area*). Both representations are on the same timescale (top). *Arrow* indicates a 50 nM Ca^{2+} gradient in the direction of the nucleus (highest around nucleus). Results are representative of 4 experiments on gonadotropes.

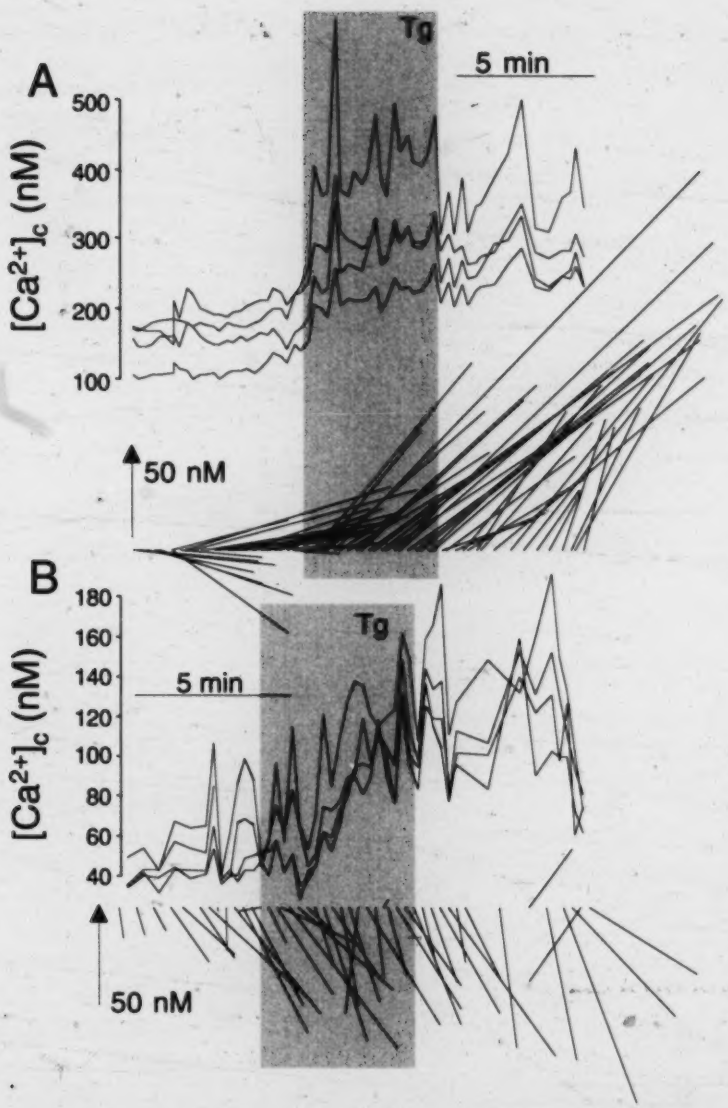

Figure 3.3. Modulation of basal Ca^{2+} gradients by thapsigargin (Tg)-sensitive Ca^{2+} stores. Spatial $[Ca^{2+}]_c$ levels in an identified gonadotrope (*top*) and somatotrope (*bottom*) are expressed at quadrant traces and vectors before, during and after exposure to 2 M Tg (*gray area*). *Arrow* indicates a 50 nM Ca^{2+} gradient in the direction of the nucleus. Results are representative of 13 of 20 experiments on gonadotropes and 3 of 7 experiments on somatotropes.

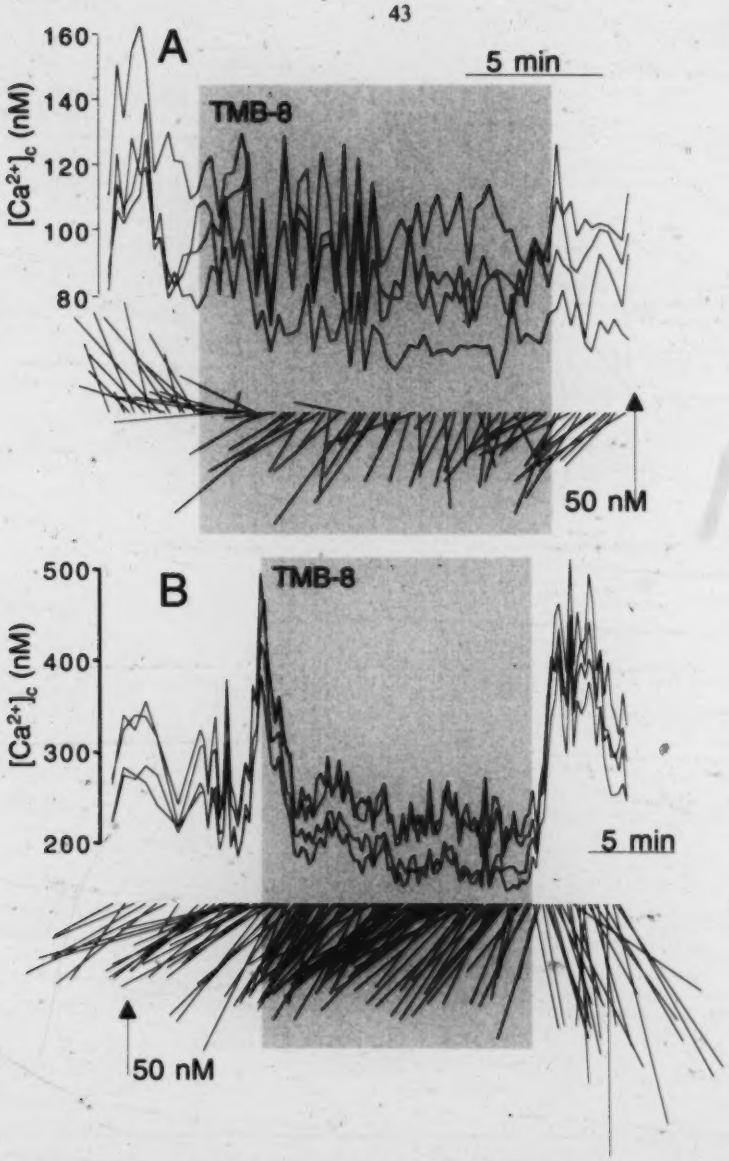

Figure 3.4. Modulation of basal Ca^{2+} gradients by intracellular Ca^{2+} release. Spatial $[Ca^{2+}]_c$ levels in an identified gonadotrope (*top*) and somatotrope (*bottom*) are expressed at quadrant traces and vectors before, during and after exposure to 100 M TMB-8 (*gray area*). *Arrow* indicates a 50 nM Ca^{2+} gradient in the direction of the nucleus. Results are representative of 7 of 9 experiments on gonadotropes and 6 experiments on somatotropes.

Chapter 4.

Agonist-specific Ca^{2+} signals in goldfish pituitary cells*

As with many secretory systems, GnRH stimulation of gonadotropin secretion is mediated by Ca^{2+} signals in all vertebrate models examined (Stojilkovic and Catt, 1995; Jobin and Chang, 1992; Mollard and Kah, 1996; Bosma et al., 1997). In mGnRH-stimulated rat gonadotropes, oscillatory and non-oscillatory temporal profiles, as well as localized, rapidly-propagating Ca^{2+} waves have been described (Tse et al., 1993; Stojilkovic and Catt, 1995; Leong and Thorner, 1991; Rawlings et al., 1991). Other secretagogues, such as endothelin, can produce Ca^{2+} signal waveforms that are distinct from those induced by GnRH (Stojilkovic et al., 1992a), demonstrating that agonist-specific Ca^{2+} signals exist in gonadotropes, as in other cell types (Petersen et al., 1991).

In a number of teleost species, including goldfish, multiple GnRH forms are delivered to the pituitary (Yu et al., 1991) where they regulate luteinizing hormone-like maturational GTH-II secretion (Chang et al., 1996). Whether different endogenous GnRH neuropeptides utilize identical or distinct signal transduction mechanisms to stimulate GTH secretion is not well understood in most cases, but has been most extensively studied in the goldfish (Chang et al., 1996). Recently, short (10s) applications of sGnRH and cGnRH-II were reported to induce either rapid/transient or slow/longer-lasting elevations in $[Ca^{2+}]_c$ in goldfish gonadotropes (Mollard and Kah, 1996). However, a detailed spatial and temporal characterization and comparison of sGnRH- and cGnRH-II-generated Ca^{2+} signals, and their concentration-dependency has not been carried out.

Although available data suggests that both GnRH peptides act on goldfish gonadotropes through a single population of receptors (Murthy et al., 1993; Habibi et al., 1990; Murthy and Peter, 1994; Yu et al., 1998), sGnRH and cGnRH-II signal transduction mechanisms differ in their relative dependence on intracellular and extracellular Ca^{2+} availability, inositol phosphate production, AA mobilization and protein kinase C activation (Jobin and Chang, 1992; Chang et al., 1996; Chang et al., 1995; Chapter 2). These signal transduction components may be regulated by Ca^{2+}, and are known to modulate Ca^{2+} fluxes in many cells, including rat gonadotropes (Stojilkovic and Catt, 1995; Noar et al., 1998; Tse et al., 1997). This raises the intriguing possibility that sGnRH- and cGnRH-II-evoked Ca^{2+} signals may have spatial and temporal differences arising from, or causing, differences in their signal transduction pathways.

In the present study, we used Fura-2 $[Ca^{2+}]_c$ imaging to investigate possible differences in Ca^{2+} signals evoked by 5-minute treatments of sGnRH or cGnRH-II in single identified goldfish gonadotropes. Spatial and temporal patterns of Ca^{2+} signals were examined. Both sGnRH- and cGnRH-II-stimulated Ca^{2+} signals were analyzed quantitatively and found to differ in their respective rates of rise, but not in other temporal characteristics. This is the first report, to

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* Components of this chapter have been published in: Johnson JD, Van Goor F, Wong CJH, Goldberg JI, Chang JP., 1999. Two endogenous gonadotropin-releasing hormones generate dissimilar Ca^{2+} signals in identified goldfish gonadotropes. Gen. Comp. Endocrinol. 116, 178-191.

suggest that two closely-related endogenous peptides act on a single receptor population to generate unique Ca^{2+} signals.

Results

Resting $[Ca^{2+}]_c$ in identified gonadotropes

Resting $[Ca^{2+}]_c$ levels, recorded from 141 identified gonadotropes, averaged 226 ± 13 nM. In unstimulated gonadotropes, $[Ca^{2+}]_c$ remained relatively constant (average coefficient of variance 5.5%). There was no evidence of regular Ca^{2+} oscillations, even when gonadotropes were imaged at higher rates (every 4 seconds; n = 20, data not shown). These results strongly suggest that basal $[Ca^{2+}]_c$ levels are tightly regulated in individual gonadotropes.

GnRH generates Ca^{2+} Signals

Both sGnRH and cGnRH-II generated rapid and reversible Ca^{2+} signals (Fig. 4.1). GnRH-evoked Ca^{2+} signals could be repeated in the same cell (Fig. 4.1 A,B). We have previously shown that GnRH-evoked Ca^{2+} signals increase does-dependently, when quantified using maximal amplitude (Johnson et al., 1999). In addition to the amplitude, the rate of rise (quantified from application of GnRH and onset of response; Fig. 4.2) and the average Ca^{2+} increase (data not shown) were also concentration-dependent. There was a trend for cGnRH-II-stimulated Ca^{2+} signals to rise faster than those of sGnRH at higher concentrations; differences were significant at GnRH concentration of 100 nM (Fig. 4.2). Concentration-dependence of the spatial characteristics was not noted.

Specificity of GnRH Ca^{2+} signalling in identified cells

Mixed populations of dispersed pituitary cells contain several cell types. Of these, gonadotropes and somatotropes are known to be regulated by GnRH (Chang et al., 1996). To test whether other pituitary cell types also respond to GnRH with Ca^{2+} signals, cells that did not fit the morphological profile(s) of either gonadotropes or somatotropes (Van Goor et al., 1994) were challenged with 5-minute pulses of 100 nM sGnRH or cGnRH-II. Of these cells, 95% showed no significant GnRH-induced increase in $[Ca^{2+}]_c$, but could be stimulated by depolarization with KCl (30 mM; n=19). Moreover, out of a total of 45 identified gonadotropes subjected to 5-minute applications of either 100 nM sGnRH or cGnRH-II, only 2 cells did not respond significantly. Similar results were observed in previous experiments using 2-minute challenges of 100 nM sGnRH and cGnRH-II, in which only 2 out of 36 cells did not respond (Johnson et al., 1999). Thus, regardless of the GnRH application protocol, greater than 95% of gonadotropes identified using these morphological characteristics responded to GnRH. This success rate compares favorably with the reported accuracy rate for the identification of goldfish gonadotropes in mixed culture (94% accuracy; Van Goor et al., 1994). Taken together, these findings lend further support to the accuracy and reliability of our cell identification method.

Quantitative comparison of Ca^{2+} signals between sGnRH and cGnRH-II

Subjectively placing responses into categories by temporal profile, although commonly practiced, has the limitation of being qualitative. Therefore, we elected to quantitatively compare the Ca^{2+} signals generated in sGnRH- (n=24) and cGnRH-II- (n=19) stimulated gonadotropes (Table 4.1). Several features of the Ca^{2+} signals were considered. The latency was relatively short and statistically the same for sGnRH and cGnRH-II. Likewise, the time to maximal amplitude did not differ statistically between the two peptides (sGnRH: 122 ± 15 seconds, cGnRH-II: 90 ± 20 seconds). In previous experiments using 2-minute challenges, the maximal amplitude of Ca^{2+} signals was similar between sGnRH and cGnRH-II over a dose range of 0.001 to 100 nM (Johnson et al., 1999). Accordingly, the maximal amplitude above baseline did not differ significantly between Ca^{2+} signals generated by 100 nM sGnRH and 100 nM cGnRH-II in the present study (Table 4.1). Similarly, when normalized to pretreatment Ca^{2+} levels, maximal amplitude did not differ significantly between sGnRH (202 ± 17 %) and cGnRH-II (184 ± 23 %). The average Ca^{2+} increase also did not differ between sGnRH- and cGnRH-II-induced Ca^{2+} signals. In contrast, the rate of rise of cGnRH-II-evoked Ca^{2+} signals, was significantly faster than that of sGnRH-stimulated Ca^{2+} signals. This difference was observed when the rate of rise was calculated either from the application of GnRH or from the onset of Ca^{2+} signals (Table 4.1). Overall, the greater rate of rise in cGnRH-II stimulated gonadotropes is the result of a reduced time to reach maximal amplitude, rather than a difference in maximal amplitude.

Classification of GnRH-induced Ca^{2+} signals by waveform

To facilitate comparison with previous reports, Ca^{2+} signal waveforms from cells stimulated for 5 minutes with maximal concentrations of GnRH were also qualitatively categorized into biphasic, monophasic, or spiking patterns (Fig. 4.3). Biphasic profiles, similar to those reported for rat gonadotropes (Thomas and Waring, 1997; Stojilkovic and Catt, 1995), were the most common waveform. These were observed in 46% of sGnRH- and 42% of cGnRH-II-induced responses. Monophasic profiles, which were typically slow-rising, accounted for 42% and 21% of the responses to sGnRH and cGnRH-II, respectively. Other responses, which typically had waveforms suggestive of oscillations and/or spiking were also observed (12% sGnRH, 37% cGnRH-II). Fluctuations were occasionally seen superimposed on other waveforms. Generally, regular and robust oscillations, such as those required for frequency coding of Ca^{2+} signals, were not observed. Therefore, the results of this investigation suggest that $[Ca^{2+}]_c$ oscillations are a minor component in the signal transduction GnRH peptides in goldfish gonadotropes.

Total response duration was not quantified because slower collection rates, used after the drug application, did not afford the temporal resolution needed to accurately determine the termination of the response. Notwithstanding, it was noticed that monophasic responses often persisted, sometimes for longer than ten minutes at levels above the pre-challenge baseline (data not shown). In biphasic profiles, the signal generally lasted only two minutes following the end of GnRH application (data not shown). Persistence of the Ca^{2+} signal, in the absence of

GnRH, was not normally observed in cells exhibiting spiking responses.

Classification of GnRH-induced Ca^{2+} signals by spatial pattern

During a previous characterization of concentration-dependent Ca^{2+} signals evoked by 2-minute pulses of GnRH, it was noted that a majority of gonadotropes (18 of 23) challenged with 100 nM sGnRH or cGnRH-II generated spatially localized increases in $[Ca^{2+}]_c$ (Johnson et al., 1999). Of the 43 gonadotropes responding to 5-minute application of 100 nM GnRH in the present study, 42 were suitable for spatial analysis and classification into either global or localized Ca^{2+} signals (one response could not be unequivocally categorized). Both GnRHs stimulated global and localized Ca^{2+} signals (Fig. 4.4). Similar to results with 2-minute applications, localized elevations in $[Ca^{2+}]_c$ comprised 57% of sGnRH- (n=23) and 53% of cGnRH-II- (n=19) induced Ca^{2+} signals in experiments using 5-minute challenges. The position of the nucleus was noted and used as a cellular landmark. Among gonadotropes responding to a 5-minute challenge with localized Ca^{2+} signals, responses that were proximal to the nucleus occurred in 85% and 60% of cells stimulated by 100 nM sGnRH or 100 nM cGnRH-II, respectively.

In cases when localized $[Ca^{2+}]_c$ increases were detected, they remained in these areas of the cell throughout the duration of the response (e.g. Fig. 4.5). These sub-cellular regions often displayed higher resting Ca^{2+} levels, creating clear spatially-defined Ca^{2+} gradients (even in unperifused cells; see Chapter 3). Ca^{2+} waves that propagate across the focal plane of the cell image, such as those reported for other cell types, including rat gonadotropes (Rawlings et al., 1991), were not observed. Moreover, Ca^{2+} signals that covered the entire plane of focus remained global throughout the response (data not shown; Johnson et al., 1999). Therefore, in goldfish gonadotropes, the location of Ca^{2+} signals does not change during the response. These observations suggest that Ca^{2+} is also tightly regulated spatially in gonadotropes.

Multi-regional analysis of Ca^{2+} signals

Spatial characteristics of GnRH-induced Ca^{2+} signals were further investigated using a combined spatial and temporal analysis. Using the same images as above, $[Ca^{2+}]_c$ levels were followed in several sub-cellular locations, over time. It was found that the temporal characteristics of the whole-cell averaged Ca^{2+} signal, were also evident at the sub-cellular level, albeit at different amplitudes. For example, the Ca^{2+} signals often reached the maximal amplitude in all studied regions of the cell at the same time (during the same image; Fig. 4.5). This analysis was also useful in determining how complex temporal patterns (like 'whole-cell' biphasic Ca^{2+} signals) are generated. Since these waveforms can also be seen concurrently in multiple regions of the cell, biphasic responses can not be a combination of a transient spike in one region of the cell and a monophasic response generated in a different part of the cell that overlap in time.

We also used this technique to compare the spatial features of agonist-induced Ca^{2+} signals. The spatial distribution of the Ca^{2+} signal at its time of maximal amplitude was similar between GnRH and 30 mM KCl, which has been shown to

release GTH-II (Van Goor et al., 1998). However, 30 mM KCl often elicited $[Ca^{2+}]_c$ increases in more regions of the cell than GnRH (Fig. 4.5; n = 19). These data suggest that agonists which stimulate GTH-II secretion may activate both similar and distinct regions of gonadotropes.

PACAP generates Ca^{2+} signals

How do GnRH-stimulated Ca^{2+} signals compare to those evoked by other hypothalamic neuropeptides? To examine this question, we treated cells with 10 nM PACAP (Fig. 4.6). In comparison to GnRH, PACAP evoked Ca^{2+} signals exhibited a greater maximal amplitude (525 ± 166 nM above baseline; n=6). These data demonstrate that gonadotropes were capable of expressing high amplitude Ca^{2+} signals, some of which were oscillatory. PACAP evoked similar Ca^{2+} signals in identified goldfish somatotropes (maximum amplitude above baseline, 367 ± 110 nM; n=5).

Discussion

In this report, the spatial and temporal features of the Ca^{2+} signals produced during the activation of single identified goldfish gonadotropes by sGnRH or cGnRH-II were characterized and compared. We report here, for the first time, quantitative and qualitative differences in the Ca^{2+} signals between these two closely related endogenous neuropeptides and that the majority of the responses were non-oscillatory. Thus, I confirm the hypothesis that goldfish gonadotropes generate agonist-specific Ca^{2+} signals.

The ability of two GnRHs to stimulate increases in $[Ca^{2+}]_c$ had been reported for identified goldfish gonadotropes (sGnRH and cGnRH-II; Mollard and Kah, 1996) and African catfish gonadotropes (catfish (cf)GnRH and cGnRH-II; Bosma et al., 1997). However, detailed characterization and comparison of Ca^{2+} signals was lacking in previous studies. In the present study, there was a higher proportion (95%) of gonadotropes generating Ca^{2+} signals in response to GnRH than in previous investigations on goldfish (69%; Mollard and Kah, 1996) or African catfish (76%; Bosma et al., 1997). This likely reflects methodological differences in gonadotrope identification strategies (Bosma et al., 1997; de Leeuw et al., 1984) and criteria (Mollard and Kah, 1996), rather than actual responsiveness to GnRH. We report the average of the maximal amplitudes of the Ca^{2+} signals in all cells tested, in addition to example waveforms. Although averages were not reported in other studies, we found a similar range of Ca^{2+} signal amplitudes (100-800 nM above baseline) when compared to previous studies on gonadotropes from goldfish (Mollard and Kah, 1996) and ovariectomized rats (Stojilkovic and Catt, 1992).

Goldfish gonadotropes in the present study exhibited a moderately high resting $[Ca^{2+}]_c$, which may reflect the lost of the tonic inhibitory influence of dopaminergic neuronal projections (Van Goor et al., 1998; Chang et al., 2000). In the present investigation, goldfish gonadotropes did not normally produce spontaneous $[Ca^{2+}]_c$ oscillations like those documented by others in this cell type (Mollard and Kah, 1996) and other cell types (Sheenen et al., 1995). Gonadotropes of the African catfish, like the cells in this study, also did not

spontaneously oscillate (Bosma et al., 1997). Much of the previous work comparing agonist-specific Ca^{2+} signatures and functional coding of Ca^{2+} signals has focused on frequency modulation of $[Ca^{2+}]_c$ oscillations (Tse et al., 1993; Petersen et al., 1991). Rat gonadotropes, and other cell types, respond to increases in mGnRH concentration with an increase in oscillation frequency; each increase in $[Ca^{2+}]_c$ may correspond to an episode of exocytosis (Tse et al., 1993). However, the paucity of basal and stimulated oscillations in $[Ca^{2+}]_c$ in this system suggests that specificity may be coded through characteristics of the Ca^{2+} signal other than oscillation frequency. Clearly, in this, a primarily non-oscillating cell type, it would still be advantageous if the cell could differentially control multiple cellular functions using coded Ca^{2+} signals. The observation that PACAP, which is a less effective GTH-II secretagogue *in vitro* (JP Chang, NR Wirachowsky, P Kwong, JD Johnson, unpublished results), evoked much larger Ca^{2+} signals compared to GnRH suggests that amplitude coded Ca^{2+} signals, an obvious alternative to frequency coding, may play only a minor role in the control of GTH-II secretion (also see Chapter 10). Other features of the GnRH-evoked Ca^{2+} signals may therefore be important in the control of GTH-II secretion. The rate of rise and average $[Ca^{2+}]_c$ both increased with increasing concentration. Using these parameters, both GnRHs had an ED_{50} in the nanomolar range, with cGnRH-II being slightly more potent. This agrees with ED_{50} estimates for GnRH-stimulated GTH-II release (Khakoo et al., 1994), and GnRH receptor binding (Habibi et al., 1990).

The Ca^{2+} signal waveform, another parameter that may be important in cellular regulation, also appeared to be concentration-dependent (Johnson et al., 1999). Higher concentrations of GnRH produced more biphasic responses, a phenomenon that has also been reported for rat gonadotropes (Stojilkovic and Catt, 1992). Since biphasic responses have a faster rate of rise (Johnson et al., 1999), the concentration-dependence seen in the relative incidence of Ca^{2+} signal waveforms can be accounted for by the concentration-dependence of the rate of rise. However, the faster rate of rise seen in Ca^{2+} signals generated by cGnRH-II is not simply a result of disproportionate numbers of monophasic responses. Analysis of the Ca^{2+} responses to sGnRH and cGnRH-II in the present study revealed that a significantly faster rate of rise of cGnRH-II-stimulated Ca^{2+} signals is still seen when only biphasic responses are considered (data not shown).

In the present investigation, maximal concentrations of both GnRH neuropeptides produced several distinct Ca^{2+} signal waveforms. Variability in Ca^{2+} signal waveforms has also been reported in rat gonadotropes (mGnRH; Tse et al., 1993; Thomas and Waring et al., 1997; Leong and Thorner, 1991; Stojilkovic and Catt, 1992), somatotropes and lactotropes (TRH; Lorsignol et al., 1997). The most common profiles in goldfish gonadotropes were biphasic Ca^{2+} signals, observed in over 40% of goldfish gonadotropes challenged with either 100 nM sGnRH or 100 nM cGnRH-II. The prevalence of biphasic Ca^{2+} signals agrees with studies conducted on African catfish gonadotropes (Bosma et al., 1997). In sGnRH-stimulated cells, monophasic responses were the next most common temporal profile; in contrast, these were the least common in cGnRH-II-stimulated gonadotropes. These results suggest that sGnRH- and cGnRH-II-

stimulated Ca^{2+} signals also differ in their ability to generate multiple Ca^{2+} signal waveforms. However, we cannot rule out the possibility that the differences in waveforms are attributable to different populations of gonadotropes.

Resting and agonist-stimulated goldfish gonadotropes often display $[Ca^{2+}]_c$ gradients that persist over time. During localized responses, some areas of the cell were completely unaffected by the GnRH-generated Ca^{2+} signal. These observations indicate that Ca^{2+} buffering systems tightly regulate Ca^{2+} levels in space. Because many cellular functions are compartmentalized spatially within cells (reviewed in Chapter 1), the potential for multiple regions to act independently of each other is a prerequisite for spatial Ca^{2+} signal coding. The possible mechanisms of these maintained Ca^{2+} gradients are further discussed in Chapter 10. Often, areas with higher resting levels of free Ca^{2+} also responded to GnRH and KCl, and tended to generate local Ca^{2+} signals of higher amplitude. These areas may be putative 'active zones' or 'trigger zones' from which Ca^{2+} signals are initiated (Clapham, 1995; Bootman et al., 1997). The higher resting Ca^{2+} may bring these regions closer to the threshold for Ca^{2+}-induced Ca^{2+} release, thereby promoting the initiation of Ca^{2+} signals. Whether, spatially discrete increases in $[Ca^{2+}]_c$ arise from a concentration of membrane Ca^{2+} channels or localized intracellular Ca^{2+} stores, or both, remains to be elucidated.

Although the time to maximal amplitude was similar in all regions measured, the regional latency was often different. In almost all cases, significant responses could be observed in the areas including the 'active/trigger zone' immediately after GnRH application. These observations suggests that our latency measurement represents the time that a Ca^{2+} signal takes to propagate throughout a large enough cross-section of the cell that it can significantly change the cellular average Ca^{2+} measurement. Rapidly-propagating Ca^{2+} waves, like those observed in rat gonadotropes (approximately 80 µm/sec; Rawlings et al., 1991), could not be detected at the temporal resolution used in the present study. Further studies using higher time resolution will be required to study the possibility that Ca^{2+} waves exist in goldfish gonadotropes.

Quantitative analysis of agonist-generated Ca^{2+} signals is a powerful tool for the study and comparison of the mechanisms and functional consequences of differently-coded Ca^{2+} signals. Using this approach, the present study demonstrates that two closely-related neuropeptides generate quantitatively distinct Ca^{2+} signals. The rate of rise of the Ca^{2+} signals may be physiologically important in determining to what degree Ca^{2+} buffers and extrusion systems are overcome or activated by the elementary increases in Ca^{2+} which combine to form Ca^{2+} signals (Clapham, 1995; Bootman et al., 1997; Hernandez-Cruz et al., 1997). Recently, neuronal Ca^{2+}-induced Ca^{2+} release was only triggered by Ca^{2+} signals with a fast rate of rise (Hernandez-Cruz et al., 1997). If Ca^{2+}-induced Ca^{2+} release is responsible for the propagation of Ca^{2+} signals in goldfish gonadotropes, the rate of rise could dictate the spatial and temporal characteristics of the Ca^{2+} signal and consequently modulate GTH-II secretion, as well as other cellular signal transduction components.

The finding that sGnRH and cGnRH-II induced disparate proportions of temporal profiles and kinetically different Ca^{2+} signals, coupled with previous

observations that the two GnRHs use partially dissimilar second messenger systems (Chang et al., 1996), raises questions regarding the mechanisms of these differences. Ca^{2+} signals are generated in response to both GnRHs when applied sequentially to the same gonadotrope (Fig. 4.1), a result that argues against the existence of sGnRH- or cGnRH-II-specific gonadotrope subpopulations. The mechanism by which two closely-related peptides act on the same population of receptors, leading to different Ca^{2+} signals remains to be elucidated. One possibility is that sGnRH and cGnRH-II, which have slightly different affinities for the goldfish gonadotrope GnRH receptor (sGnRH: $K_a = 3.3 \pm 0.9\ 10^9\ M^{-1}$, cGnRH-II: $K_a = 9.2 \pm 0.1\ 10^9\ M^{-1}$; Habibi et al., 1990), may induce distinct receptor conformations. Many seven-transmembrane domain receptors, including the rat GnRH receptor (Stanislaus et al., 1998) have been shown to couple to more than one type of G-protein in the same cell type. It is conceivable that agonist-specific conformations of the goldfish gonadotrope GnRH receptor could lead to the differential association with, and activation of, distinct G-proteins. The increased rate of rise of cGnRH-II-evoked Ca^{2+} signals may reflect a signal transduction cascade that is thought to be more reliant on influx of Ca^{2+} through VGCC, in comparison to that of sGnRH (Chang et al., 1996). In principle, propagation of Ca^{2+} signals by modulating fluxes from intracellular Ca^{2+} stores might be slower than the VGCC-mediated signalling commonly found in excitable cells (e.g. Zucker and Fogelson, 1986). Given that both GnRHs elicit secretion of GTH-II in goldfish, the need for GnRH-specific kinetics and multiple Ca^{2+} signals generated by each peptide is unclear. Different GnRH-stimulated Ca^{2+} signals could target distinct cellular functions (Leong and Thorner, 1991). It has been reported that sGnRH is more consistently effective than cGnRH-II in increasing GTH-II β subunit levels in goldfish (Khakoo et al., 1994). Whether slower or monophasic Ca^{2+} signals, which are more common among sGnRH-evoked responses, lead to this difference remains to be investigated. The duration of Ca^{2+} signals has been shown to be important in the regulation of gene expression in several cell types (Dolmetsch et al., 1997). In addition, a longer Ca^{2+} signal duration was recently shown to be crucial in the activation of AA production by phospholipase A_2 (Hirabayashi et al., 1999). In goldfish gonadotropes, AA is only involved in sGnRH signalling (Chang et al., 1996). Whether each type of Ca^{2+} signal leads to GTH-II synthesis and/or exocytosis in every gonadotrope remains to be determined.

Although multiple GnRHs are present in most vertebrates studied (King and Millar, 1995), including primates and other mammals (White et al., 1997; Lescheid et al., 1997) very little is known about the diversity of their signal transduction, including their Ca^{2+} signalling. The present study provides a comprehensive account of the spatial patterns, temporal profiles and kinetic features of the Ca^{2+} signal generated by sGnRH and cGnRH-II. We show here, for the first time, kinetic differences in the Ca^{2+} signals of two closely related neuroendocrine regulators which are thought to act through the same population of receptors.

TABLE 4.1. Quantitative comparison of Ca^{2+} signals in gonadotropes stimulated for 5 minutes with either 100 nM sGnRH or 100 nM cGnRH-II as calculated in Chapter 3. All values are presented as mean ± SEM.

Treatment‡	Kinetic Parameter				
	Latency§ (s)	Rate (Appl.) (nM/s)	Rate (Onset) (nM/s)	Max. Ampl. (nM)	Average Ca^{2+} (nM)
sGnRH	19 ± 3	1.6 ± 0.3	2.0 ± 0.3	154 ± 25	95 ± 10
cGnRH-II	21 ± 3	5.2 ± 1.5*	7.2 ± 2.0*	198 ± 43	95 ± 20

‡ number of independent trials for sGnRH and cGnRH-II were 24 and 19, respectively.
* denotes significant difference compared to sGnRH using a Student's *t*-test ($P < 0.05$).
§ note that each latency value includes an 'extra' 15 seconds (see Chapter 3 for rational).

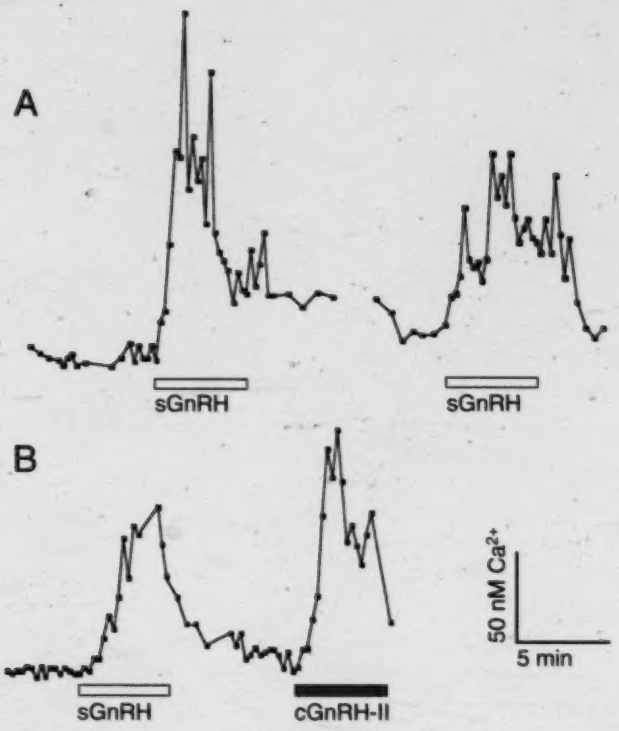

Figure 4.1. GnRH generates Ca^{2+} signals in identified gonadotropes. A) Sequential challenges with sGnRH (*open bars* throughout Figure) evoke Ca^{2+} signals in identified gonadotropes (n=6). Resting $[Ca^{2+}]_i$ of this representative trace was ~110 nM. Break in trace indicates 5 minutes elapsed time. B) sGnRH and cGnRH-II (*gray bars* throughout Figure) evoke Ca^{2+} signals in the same gonadotrope (n=7). Resting $[Ca^{2+}]_i$ of this representative trace was ~ 80 nM. Scales bars indicate Ca^{2+} levels and time for both panels.

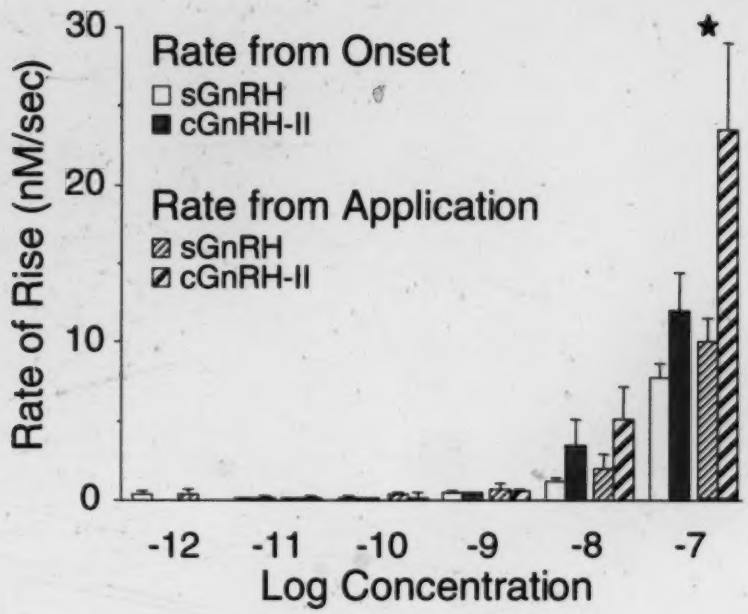

Figure 4.2. The rate of rise of GnRH-evoked Ca^{2+} signals is concentration-dependent in identified gonadotropes. Rates of rise were calculated from the onset of the GnRH-evoked Ca^{2+} signals (sGnRH: *solid white bars*; cGnRH-II: *solid dark bars*) and from the beginning of the 2-minute applications of GnRH (sGnRH: *light stripes*; cGnRH-II: *heavy stripes*). Analysis of rate of rise was preformed on data found in Johnson et al., 1999. Ca^{2+} signals possessed a significantly faster rate of rise from the application of 100 nM cGnRH-II, when compared to 100 nM sGnRH (Mann-Whitney U, $P < 0.05$).

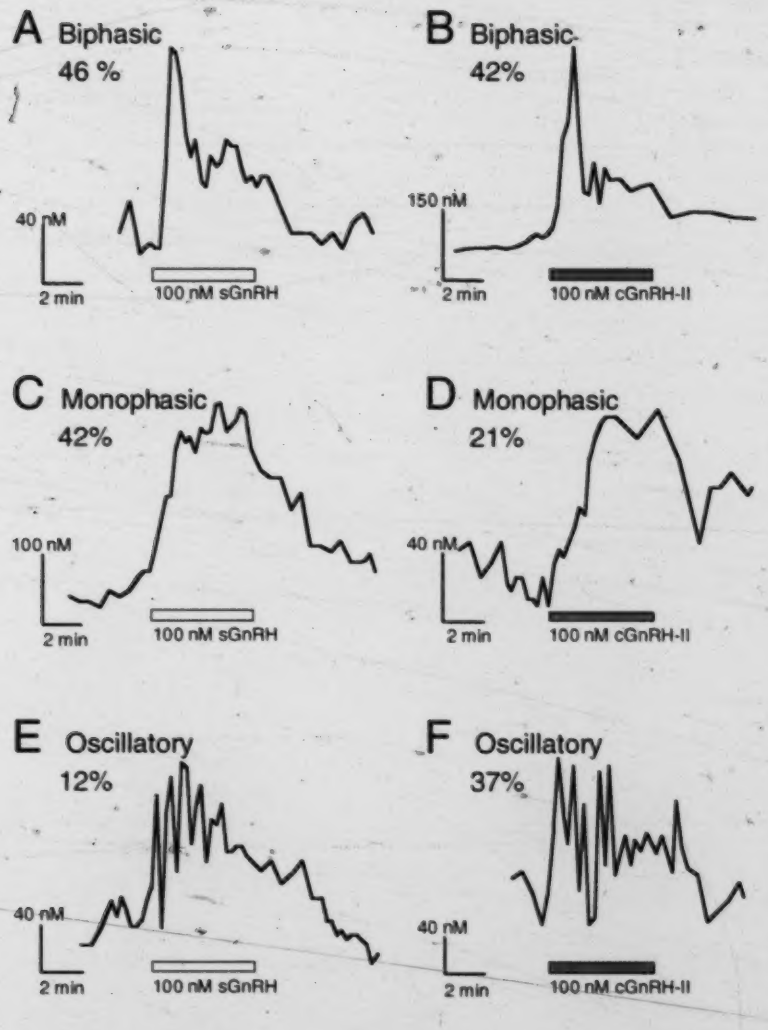

Figure 4.3. Both GnRH peptides generate multiple Ca^{2+} signal waveforms. sGnRH (A,C,E) and cGnRH-II (B,D,F) both produce Ca^{2+} signals of three temporal profiles when applied as 100 nM 5-minute challenges to identified gonadotropes. Shown are example traces, with the corresponding percentage of total responses, of biphasic Ca^{2+} signals (A,B), monophasic Ca^{2+} signals (C,D), and spiking Ca^{2+} signals (E,F) induced by 5-minute application of GnRH (n=24 sGnRH; n=19 cGnRH-II). Images were acquired every 15 seconds during the GnRH challenge period. Horizontal bars indicate the duration of GnRH application.

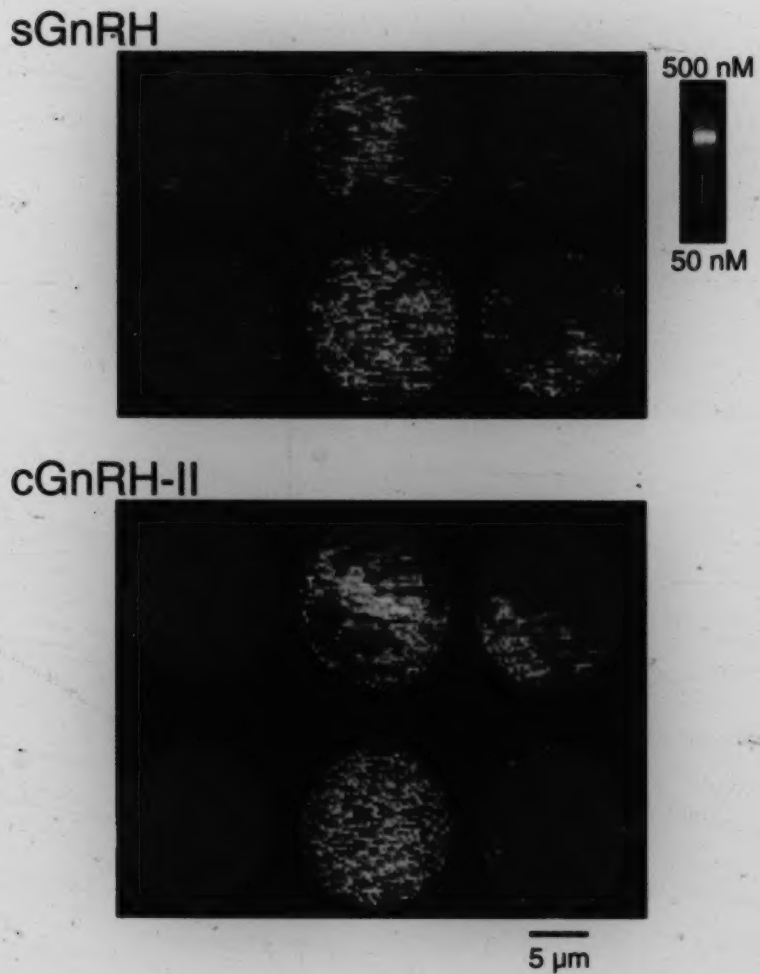

Figure 4.4. Spatial features of Ca^{2+} signals evoked by 100 nM sGnRH or 100 nM cGnRH-II. Fura-2 loaded gonadotropes are shown before (*left*), at the peak of the GnRH response (*center*) and after washout of GnRH (*right*).

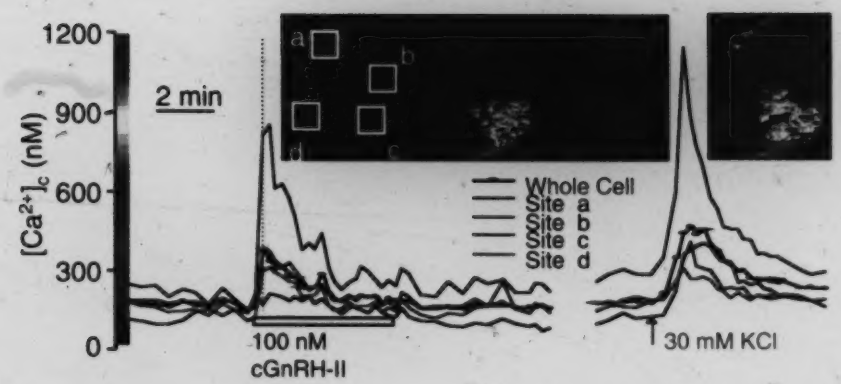

Figure 4.5. Multi-region temporal analysis of GnRH-evoked Ca^{2+} signals. A representative GnRH-generated Ca^{2+} signal is analyzed in the sub-cellular regions (*colored lines*) indicated by the white squares in the first (left) image. Yellow bar indicates the application of 100 nM cGnRH-II. The spatial and temporal features of cGnRH-II-generated Ca^{2+} signal are compared with that of 30 mM KCl depolarization (*red arrow*). The dotted orange line indicates the time of maximal amplitude of the whole cell recording (*black line with filled circles*). Note that all regions of the cell measured achieved maximal amplitude at approximately the same time, but differed considerably in their maximal amplitude. ***Inset***, images (*left to right*) are resting, at the maximal amplitude of cGnRH-II-generated Ca^{2+} signal stimulation, washout, and at the maximal amplitude of a subsequent 30 mM KCl-induced Ca^{2+} signal in the same cell. Note the differences in the spatial distribution of the Ca^{2+} signal between GnRH and KCl. Data shown are representative of 10 independent experiments.

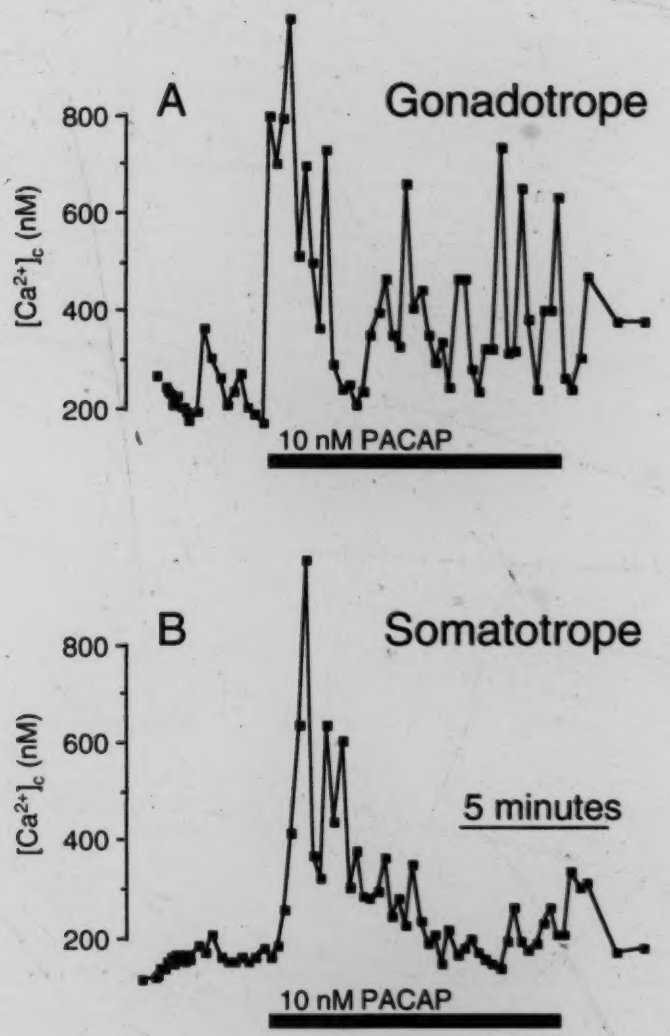

Figure 4.6. PACAP generates Ca^{2+} signals in gonadotropes and somatotropes, 10 nM PACAP was applied as indicated (*black bars*). Experiments were repeated on 6 gonadotropes and 5 somatotropes with similar results.

Chapter 5.

Agonist-specific Ca^{2+} signalling systems composed of multiple intracellular Ca^{2+} stores regulate gonadotropin secretion*

The functional specificity of Ca^{2+}-dependent signal transduction may be determined by unique spatial and temporal features of the Ca^{2+} signals generated by agonists (Bading et al., 1993; for reviews see Berridge, 1997; Crabtree, 1999; Johnson and Chang, 2000a; Chapter 1). Chapter 4 described the generation of agonist-specific Ca^{2+} signals in goldfish gonadotropes by two endogenous gonadotropin-releasing hormones, which are thought to act through a single type of receptor; the Ca^{2+} signals evoked by cGnRH-II had a faster rate of rise than those elicited by sGnRH. This result complemented previous work which has demonstrated several differences in the signal transduction and biological activity of these two peptides (Chang et al., 1995; Khakoo et al., 1994; reviewed in Chang et al., 1996). Importantly, the relative importance of extracellular Ca^{2+} availability on GTH-II-releasing activity was found to be different between sGnRH and cGnRH-II (Jobin and Chang, 1992; Chang et al., 1995). Taken together, these findings suggested that the processes underlying the Ca^{2+} signals generated by sGnRH and cGnRH-II may be distinct.

To date, studies have indicated that both sGnRH- and cGnRH-II-stimulated GTH-II release is highly dependent on extracellular Ca^{2+} entry (Jobin and Chang, 1992; Jobin et al., 1996) through VGCC present on the gonadotrope plasma membrane (Van Goor et al., 1996a). However, pharmacological inhibitors of VGCCs were delivered to cells far in advance of GnRH treatment in those previous studies. Since active intracellular Ca^{2+} stores can be depleted within minutes when access to extracellular Ca^{2+} is interrupted (Van Goor et al., 1999; Tse, 1993), such a maneuver is likely to have emptied rapidly exchanging, agonist-sensitive intracellular Ca^{2+} stores. Thus experiments conducted in such a manner cannot distinguish whether the source of Ca^{2+} mobilized during acute GnRH-signal transduction is intracellular or extracellular. In rat gonadotropes, acute mGnRH-stimulated of GTH release is entirely dependent on IP_3-sensitive intracellular Ca^{2+} stores, while extracellular Ca^{2+} is only required for the refilling of endoplasmic reticulum Ca^{2+} stores (Tse et al., 1993; Hille et al., 1995).

Here, pharmacological manipulations are used to show that intracellular Ca^{2+} stores mediate GnRH-stimulated Ca^{2+} signals and GTH-II secretion in goldfish gonadotropes, but that extracellular Ca^{2+} influx may be linked to GTH-II release through a novel homeostatic mechanism. Most importantly, by comparing the relative involvement of multiple intracellular Ca^{2+} pools in the signal transduction of the two GnRHs we provide the first demonstration that two closely related endogenous neuropeptides use agonist-specific Ca^{2+} signalling systems.

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* A version of this chapter is in press: Johnson JD, Van Goor F, Jobin RM, Wong CJH, Goldberg JI, Chang JP. 2000. Agonist-specific Ca^{2+} signalling systems, composed of multiple intracellular Ca^{2+} stores, regulate gonadotropin secretions. Mol. Cell Endocrinol.

Results

Previous experiments have shown that, in spite of long incubations with nominally Ca^{2+}-free media or VGCC blockers, significant levels of acute GnRH-stimulated GTH-II release still persists (e.g. Jobin et al., 1996). Does this represent a Ca^{2+}-independent component of GnRH-stimulated GTH-II release or Ca^{2+} released from intracellular sources? To test the former possibility, we loaded cells with BAPTA by pre-incubating cultures with 50 µM BAPTA-AM for 40 minutes. Intracellular BAPTA is known to inhibit the generation of transient Ca^{2+} signals without greatly affecting baseline $[Ca^{2+}]_c$ in all cell types tested, including rat gonadotropes (even at concentrations higher than those used in the present study; Masumoto et al., 1995). Although basal GTH-II secretion was unaffected, GnRH-evoked release was completely abolished in BAPTA loaded cells (Fig. 5.1). These findings demonstrate a critical dependence on the generation of Ca^{2+} signals, but do not indicate the source of the Ca^{2+} explicitly. Having eliminated the possibility that GnRH-stimulated GTH-II release contains a Ca^{2+}-independent component, we turned our attention to the involvement of intracellular Ca^{2+} stores in acute GnRH-stimulated Ca^{2+} signals leading to GTH-II release.

Mobilization of intracellular Ca^{2+} pools triggers GnRH-stimulated GTH-II release

To directly examine the involvement of intracellular Ca^{2+} stores in gonadotrope signal transduction, we used several specific pharmacological manipulations. Treatment of cells with 100 µM TMB-8, an extensively used, broad-spectrum inhibitor of intracellular Ca^{2+} channels (Malgodi and Chiou, 1974; Chini and Dousa, 1996; Anderson et al., 1992), completely abolished Ca^{2+} signals generated by both sGnRH and cGnRH-II (Fig. 5.2). Subsequent challenges with 30mM KCl still evoked Ca^{2+} signals. Interestingly, 3 of 8 cells responded to TMB-8 with a steady increase in $[Ca^{2+}]_c$, which may reflect a homeostatic over-compensation from other Ca^{2+} sources. Importantly, acute GnRH-stimulated GTH-II release was completely abolished in the presence of TMB-8 when compared to both parallel and sequential GnRH controls (Fig. 5.3). Note that the quantified net GTH-II response to GnRH in the presence of TMB-8 was not different from the background secretory activity during the same period in cells exposed to TMB-8 alone. The inhibition of GnRH-stimulated GTH-II release was not due to the blockade of events subsequent to Ca^{2+} mobilization, since 100 µM TMB-8 had little or no effect on GTH-II release evoked by 10 µM ionomycin, 1 µM TPA or 10 µM forskolin (Johnson et al., 2000).

Another method of directly evaluating the role in intracellular stores is to prevent their Ca^{2+} loading with selective inhibitors of the SERCA family of intracellular Ca^{2+}ATPases, such as CPA and BHQ (Mason et al., 1991). Pretreatment of perifused cells with either of these structurally-unrelated compounds resulted in significant inhibition of GnRH-stimulated GTH-II release (10 µM CPA, Fig.5.4). Similarly, significant GTH-II responses to sGnRH and cGnRH-II could not be observed in the presence of 10 µM BHQ ($P > 0.05$ vs zero

response, t-test, n=6 for each GnRH; not shown). Thus, CPA- and BHQ-sensitive SERCA pumps may be necessary in the control of regulated GTH-II release.

IP_3 is the prototypical link between receptor activation and intracellular Ca^{2+} mobilization in most cell types (Pozzan et al., 1994; Berridge, 1997). Although previous studies have suggested the possibility that goldfish GnRHs may generate IP_3 (Chang et al., 1995; Illing et al., 1999), the involvement of IP_3-sensitive intracellular Ca^{2+} stores in hormone release has not been demonstrated in goldfish pituitary cells. We used xestospongin C, a potent and selective, membrane-permeant blocker of IP_3 receptors (Gafni et al., 1997), to evaluated the role of IP_3 receptors in acute sGnRH- and cGnRH-II-stimulated GTH-II release. GnRH responses (15 minutes) were quantified from fast-fraction (1 minute/fraction) perifusion experiments. Treatment with 1 μM xestospongin (for 10 minutes) had no effect on basal hormone release (data not shown), but selectively inhibited GTH-II release evoked by sGnRH, but not cGnRH-II (Fig. 5.5). These results demonstrate that although intracellular Ca^{2+} stores are required in the acute action of the two endogenous GnRHs, only sGnRH signalling requires IP_3-sensitive intracellular Ca^{2+} stores.

Next, we tested whether direct activation of Ca^{2+} release from intracellular stores can couple to GTH-II secretion using caffeine. Caffeine is known to stimulate the release of intracellular Ca^{2+} stores by sensitizing intracellular Ca^{2+} channels to ambient Ca^{2+} levels (Ehrlich et al., 1994). Treatment with 10 mM caffeine alone caused a very large, biphasic GTH-II release response (Fig. 5.6, 5.7), indicating that mobilization of intracellular Ca^{2+} is sufficient to evoke hormone release in this system. GTH-II response to a second application of caffeine 45 minutes later was negligible, suggesting that caffeine mobilized Ca^{2+} from a pool that refilled very slowly (see Fig. 6.1B). Elsewhere, we have confirmed that caffeine-stimulated GTH-II release is independent of plasma membrane ionic currents and cAMP signalling pathway (Wong CJH, Chang JP, unpublished; see Chapter 6 for further pharmacological characterization of caffeine signal transduction). We reasoned that, if GnRH and caffeine signalling were independent, then an additive GnRH-stimulated GTH-II response would still be recorded during caffeine exposure. The results clearly indicate that only cGnRH-II-evoked GTH-II responses are additive to caffeine stimulation (Fig. 5.6). These data suggest that sGnRH and caffeine may mobilize a common intracellular Ca^{2+} pool in goldfish gonadotropes. Thus, it appears that although both GnRHs utilize TMB-8-sensitive Ca^{2+} stores refilled by SERCA pumps, an additional caffeine-sensitive intracellular Ca^{2+} pool is mobilized by sGnRH. Caffeine-stimulated GTH-II release was also not blocked by TMB-8 and only partially inhibited by CPA (Fig. 5.7), suggesting a certain degree of functional independence of these Ca^{2+} stores.

Novel interactions between Ca^{2+} homeostasis and hormone release

A final set of experiments was designed to determine the involvement of extracellular Ca^{2+} entry through VGCCs in basal GTH-II release. In the present study, and in previous experiments, we noticed that manipulating the availability of extracellular Ca^{2+} often evoked a paradoxical increase in GTH-II secretion

(Fig. 5.8; Chang et al., 1995). Indeed, this response is robust and is often larger than the GnRH-evoked GTH-II response, a fact that precluded direct experiments on the effects of Ca^{2+}-free conditions on GnRH-stimulated GTH-II release. Exposure to nominally Ca^{2+}-free media also results in transient, global increases in $[Ca^{2+}]_c$, but only in 22% of gonadotropes (data not shown). As was the case in previous experiments with 1 μM nifedipine (Jobin 1993; Van Goor, 1997), blockade of Ca^{2+} entry through VGCC with 50 μM Cd^{2+} also resulted in transient increases in basal GTH-II secretion (Fig. 5.8).

Discussion

Results of the present study clearly demonstrate that acute GnRH signalling in goldfish gonadotropes is dependent on the mobilization of intracellular Ca^{2+} stores. This conclusion contradicts previous reports that acute GnRH-stimulated GTH-II release is highly dependent on VGCC-mediated Ca^{2+} entry in goldfish (Jobin et al., 1996; Chang et al., 1996), but agrees with the well-accepted model that describes mGnRH signalling in rat gonadotropes (Iida et al., 1991; Tse et al., 1993; Hille et al., 1995). However, discrepancies between this data and previous goldfish gonadotrope literature can easily be explained when differences in experimental protocol are taken into account. In previous experiments, Ca^{2+}-free solutions and VGCC blockers were administered up to an hour in advance of GnRH treatment. Investigations of mammalian systems have suggested that GnRH-sensitive Ca^{2+} stores can be depleted within 10 minutes in nominally Ca^{2+}-free media (Tse et al., 1993; Van Goor et al., 1999). Accordingly, short pretreatments of Cd^{2+}, Co^{2+} and Ca^{2+}-free media did not inhibit GnRH-stimulated Ca^{2+} signals in goldfish gonadotropes in a previous study (Van Goor, 1997). In addition, neither sGnRH nor cGnRH-II affected membrane voltage or VGCC in patch-clamp experiments with goldfish gonadotropes (Johnson et al., 2000; Van Goor, 1997; CJH Wong, F Van Goor, and JP Chang, unpublished results). These findings strongly suggest that VGCC do not participate in the acute phase of GnRH signalling.

In contrast, direct pharmacological manipulation of multiple classes of intracellular Ca^{2+} stores suggested their involvement in GnRH-stimulated GTH-II release. It is noteworthy that TMB-8 completely abolished both the Ca^{2+} signals and hormone release stimulated by GnRH, whereas many previous studies have shown only a partial inhibition of acute GTH-II release with long-pretreatments of VGCC inhibitors and Ca^{2+}-free media (Chang et al., 1995; Jobin et al., 1996). In light of the conclusions indicated by our current data, a new working model of GnRH signalling in goldfish gonadotropes is presented (Fig. 5.9), where GnRH initiates acute GTH-II release via mobilization of Ca^{2+} from intracellular stores.

Goldfish gonadotropes clearly possess signalling processes linked to membrane excitability (Van Goor et al., 1996a; Van Goor et al., 1998; Johnson et al., 1999; Mollard and Kah, 1996; Van Goor, 1997). However, several lines of evidence suggest that acute GnRH-evoked Ca^{2+} signalling constitutes a separate, parallel pathway. First, previously reported data demonstrate that sGnRH (100 nM)-stimulated GTH-II release responses are additive to those induced by depolarization with 30 mM KCl (Chang et al., 1996). This manipulation is

significant because it would be expected to modulate VGCCs without depleting intracellular stores. Second, application of GnRH and KCl in the same cell leads to the production of Ca^{2+} signals which are somewhat spatially and temporally distinct (Johnson et al., 1999; Chapter 4), although they often share some regions of similarity. Third, although TTX-sensitive Na^+ channels are present on goldfish gonadotropes and may be involved in determining the kinetics of spontaneous action potentials, they are not involved in either sGnRH- or cGnRH-II-stimulated GTH-II release (Van Goor et al., 1996a, Van Goor et al., 1996b). Fourth, apamin-sensitive Ca^{2+}-activated K^+ channels, which are an integral component of mGnRH-induced membrane excitability in rat gonadotropes (Kukuljan et al., 1992) are not found in goldfish gonadotropes (Van Goor et al., 1996a). Finally, GTH-II release evoked by depolarizing concentrations of KCl and by the VGCC activator BayK 8644 are not consistently observed throughout the annual seasonal reproductive cycle (JD Johnson and JP Chang, unpublished data). During times when KCl and BayK 8644 fail to stimulate significant GTH-II release, the two GnRHs are still effective. In the present study, we provide further evidence for a dominant role for TMB-8/CPA-sensitive intracellular Ca^{2+} stores in GnRH-induced GTH-II release and Ca^{2+} signal generation. Taken together, these lines of evidence strongly suggest that goldfish gonadotropes possess multiple parallel Ca^{2+} signalling systems, including at least one linked to VGCCs and at least two coupling GnRH receptors to intracellular Ca^{2+} stores.

Contrary to the scheme proposed for many excitable cell types, it is likely that Ca^{2+} mobilization from intracellular stores might be more tightly coupled to exocytosis than influx through VGCCs in gonadotropes of both rat (Tse et al., 1993; Tse et al., 1997) and goldfish (this study). In spite of the fact that global GnRH-stimulated Ca^{2+} signals are substantially smaller in amplitude when compared to those evoked by 30 mM KCl (this thesis; Van Goor, 1997; Johnson et al., 1999; Mollard and Kah et al., 1996; Chang et al., 1996), GnRH evokes a greater GTH-II release response (Jobin et al., 1996; JD Johnson, F Van Goor and JP Chang, unpublished data). The massive hormone release-stimulating ability of caffeine is further evidence for a preferential coupling of Ca^{2+} stores to exocytosis. The mechanism of this tight coupling is still not completely understood and remains to be elucidated in goldfish gonadotropes. However, as proposed for other cell types, this coupling most likely involves high amplitude microdomains around intracellular Ca^{2+} release channels which are closely juxtaposed with secretory machinery (Neher, 1998; Tse et al., 1997; Gerasimenko et al., 1996a; also see Chapter 6).

Although the present findings assign new importance to intracellular Ca^{2+} mobilization in GTH-II release, a minor role for intracellular Ca^{2+} stores in gonadotrope function has been suggested previously. Activation of cloned goldfish GnRH receptors generates IP_3 in COS-7 cells (Illing et al., 1999). Also, several reports had demonstrated sGnRH, but not cGnRH-II, signalling cascades include an intracellular Ca^{2+} store(s) component linked to GTH-II release which was resistant to long periods of limited extracellular Ca^{2+} availability (Jobin and Chang, 1992; Chang et al., 1995). Furthermore, sGnRH, but not cGnRH-II, generates significant IP_3 from mixed cultures of goldfish pituitary cells (Chang et

al., 1995). These findings led to the speculation that sGnRH employed a depletion-resistant, IP_3-sensitive Ca^{2+} store. Results from the present study strongly support both of these hypothesis, but do not demonstrate that these two properties are manifested through a single sGnRH/caffeine/IP_3-sensitive Ca^{2+} pool. However, the colocalization of caffeine- and IP_3-sensitive Ca^{2+} stores in gonadotropes is a distinct possibility given the similar selectivity of xestospongin C and caffeine on sGnRH-stimulated GTH-II release. Although initially characterized as an activator of ryanodine receptors, caffeine is also known to inhibit IP_3 receptors and other components of the IP_3 signalling pathway (Ehrlich et al., 1994; Toescu et al., 1992). The possibility that a separate IP_3-sensitive pool is coupled, in series, to a downstream caffeine-sensitive pool through classical Ca^{2+}-induced Ca^{2+} release mechanisms may be ruled out since high concentrations of ryanodine have no effect on caffeine-stimulated GTH-II release (see Chapters 6, 8). It is also important to note that the inhibitory actions of caffeine on sGnRH signalling may not be manifested through the same mechanisms as the stimulatory effects of caffeine on hormone release. It is also possible that a single Ca^{2+} store may possess both IP_3 receptors involved in sGnRH signal transduction and a second Ca^{2+}-release channel activated by caffeine. Further work will be require to determine the extent to which caffeine modulates multiple targets in goldfish pituitary cells.

Importantly, based on hormone release experiments, results from this study suggest that the sGnRH/caffeine-sensitive intracellular Ca^{2+} stores of goldfish gonadotropes display several novel properties not normally associated with the typical rapidly-exchanging IP_3/agonist-sensitive Ca^{2+} stores found in endoplasmic reticulum of most cell types (Pozzan et al., 1994). The first distinguishing feature is the relative insensitivity to SERCA inhibitors (also see Chapters 6, 11). The second novel characteristic is the marked resistance to depletion in conditions when the availability of extracellular Ca^{2+} is compromised (Jobin et al., 1996; Chang et al., 1996). The sensitivity of sGnRH-stimulated GTH-II release to CPA/BHQ can be explained if an intracellular Ca^{2+} store with SERCA pumps is an upstream requirement for activation of the caffeine/xestospongin-sensitive component (see Fig. 5.9). We suggest that this role is played by the TMB-8-sensitive intracellular Ca^{2+} store which is common to both sGnRH/cGnRH-II signalling. The findings that cGnRH-II does not generate IP_3 (Chang et al., 1995) and that cGnRH-II-stimulated GTH-II release is unaffected by xestospongin C (this study) suggest that the common Ca^{2+} pool does not involve IP_3 receptor-mediated signalling. To date, such an agonist-sensitive intracellular Ca^{2+} store has not been identified in pituitary cells from any animal. Future experiments in the goldfish gonadotrope model may uncover additional novel features of agonist Ca^{2+} signalling leading to hormone exocytosis. Investigations of such complex interactions between multiple intracellular Ca^{2+} stores during agonist-stimulation have been reported for pancreatic acinar cells. In this exocrine cell, the coordinated action of three intracellular Ca^{2+} stores, sensitive to nicotinic acid adenine dinucleotide phosphate, IP_3 and ryanodine, participate in cholecystokinin-signal transduction leading to enzyme secretion (Cancela et al., 1999). Although the co-activation of

multiple Ca^{2+} stores may also be functionally important in endocrine cells, comparable studies are generally lacking. In light of the discovery of several new classes of intracellular Ca^{2+} stores (reviewed in Chapter 1; Johnson and Chang, 2000a; Lee, 2000), the possible involvement of novel second messengers, such as cyclic ADPribose and NAADP, in pituitary hormone release also requires further consideration. Interestingly, Ca^{2+} release from NAADP-sensitive intracellular stores can be inhibited by VGCC blockers (nifedipine, verapamil and diltiazem) and TMB-8, but not by SERCA inhibitors (Genazzani et al., 1996; Genazzani and Galione, 1996; Chini and Dousa, 1996). The possibility that sGnRH and caffeine may act on a non-ER Ca^{2+} store is discussed in Chapter 6.

The results of the present study point to complex interactions between multiple agonist-sensitive Ca^{2+} stores. Further complexity may exist with respect to the interactions between these intracellular Ca^{2+} stores and plasma membrane Ca^{2+} fluxes. Although GnRH does not directly activate VGCCs, evidence from earlier studies indicates that Ca^{2+} influx through VGCCs is partially required to refill the agonist-sensitive Ca^{2+} stores, and thus to maintain prolonged secretion (Jobin et al., 1996; Chang et al., 1996). It is tempting to speculate that dopamine, which reduces Ca^{2+} influx through VGCCs and endogenously inhibits GTH-II secretion (Van Goor et al., 1998), may act by reducing the content of intracellular Ca^{2+} stores. However, it is currently unclear how GnRH may recruit VGCCs to refill the intracellular Ca^{2+} stores without a discernible change in membrane voltage.

In addition to characterizing the nature of intracellular Ca^{2+} stores regulating GnRH-stimulated GTH-II release, we have also provided evidence for novel and physiologically-relevant links between basal Ca^{2+} homeostasis and GTH-II release. In the present study and in others, treatments which upset the equilibrium of intracellular Ca^{2+} homeostasis by drastically reducing the availability of extracellular Ca^{2+} consistently increased basal GTH-II release (Cd^{2+}, this study; nifedipine, Jobin, 1993; verapamil, Chang et al., 1995; Ca^{2+}-free, this study and Jobin, 1993). In contrast, goldfish growth hormone was elevated to a much lesser extent during the same experiments (Chapter 7; Johnson and Chang, 2000b; JP Chang, unpublished observations). These findings suggest that goldfish gonadotropes may possess a more sensitive mechanism for sensing changes in Ca^{2+} availability or that this signal is more tightly coupled to hormone release, or both. As is the case for agonist-stimulation, this strong coupling may be related to the mobilization of intracellular stores. It has recently been shown that Ca^{2+} signals evoked by cGnRH-II and nifedipine display a similar spatial distribution in goldfish gonadotropes (Johnson et al., 2000; Van Goor, 1997). Furthermore, preloading intracellular Ca^{2+} stores by maintaining perifused cells in 20 mM Ca^{2+} for a prolonged period resulted in the potentiation of the response to Ca^{2+}-free conditions (Johnson et al., 2000; Jobin, 1993). Collectively, these findings suggest that the homeostatic Ca^{2+} signal which mediates the GTH-II release response to Ca^{2+}-free media results from the mobilization of intracellular Ca^{2+} stores. Although Ca^{2+} signals appear to be initiated by intracellular stores, a component of the Ca^{2+} signals may result from the activation of plasma membrane store-operated Ca^{2+} channels (SOC), which are widely expressed in

many cell types, including rat gonadotropes (McArdle et al., 1996; Barritt, 1999). The participation of these and/or other voltage-independent Ca^{2+} entry mechanisms in Ca^{2+} homeostasis and GTH-II release remains to be elucidated in goldfish gonadotropes. Interestingly, SERCA inhibitors themselves are not consistent or effective secretagogues in goldfish gonadotropes (e.g. CPA Fig. 5.7; BHQ Chapter 6; Chapter 10), thus calling into question the role of SOC in this process. Nonetheless, how multiple intracellular Ca^{2+} stores interact with each other and with plasma membrane Ca^{2+} channels is an exciting area of inquiry for the future. Moreover, we can only speculate as to the identity of the 'Ca^{2+} sensor' (which we term the 'calcistat') responsible for the initiation of this response. The fact that nifedipine and verapamil seemed to have affected this calcistat, suggests that it is not the extracellular Ca^{2+} receptor, although the possibility that such a mechanism may account for part of the Ca^{2+}-free response requires additional examination. Furthermore, it is not known whether the activation of GTH-II release under these circumstances is related to changes in global $[Ca^{2+}]_c$, localized cytoplasmic Ca^{2+} microdomains or $[Ca^{2+}]$ in the lumens of secretory pathway organelles.

Since both sGnRH and cGnRH-II act on the same population of gonadotropes, the interactions between distinct agonist-activated signalling cascades and Ca^{2+} homeostasis are likely to be complex. We hypothesize that the dynamic properties and/or strategic location of these agonist-specific intracellular Ca^{2+} systems may be important in the independent control of multiple cellular functions, such as glycoprotein mRNA levels, by sGnRH and cGnRH-II (Khakoo et al., 1994).

Besides establishing an important role of intracellular Ca^{2+} stores in initiating GnRH action, this study is the first to suggest that agonist-specific Ca^{2+} signals can be generated by the differential involvement of specific suites of intracellular Ca^{2+} stores (Fig. 5.9). In the case of sGnRH, this may include novel caffeine/IP_3-sensitive Ca^{2+} store(s). This conclusion carries added novelty since sGnRH and cGnRH-II are thought to act through the same receptor subtype.

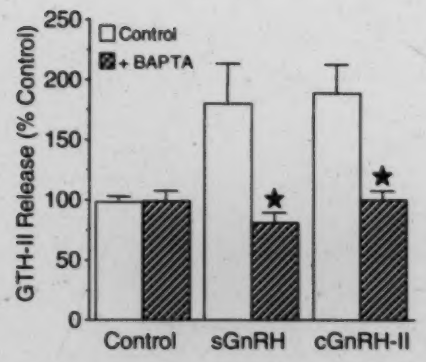

Figure 5.1. GnRH-stimulated GTH-II release requires Ca^{2+} signals. 2-hour GTH-II release responses to 100 nM sGnRH and 100 nM cGnRH-II are abolished in cells pre-incubated with 50 μM BAPTA-AM (*hashed bars*). *Star* indicates significant difference from control (n = 12).

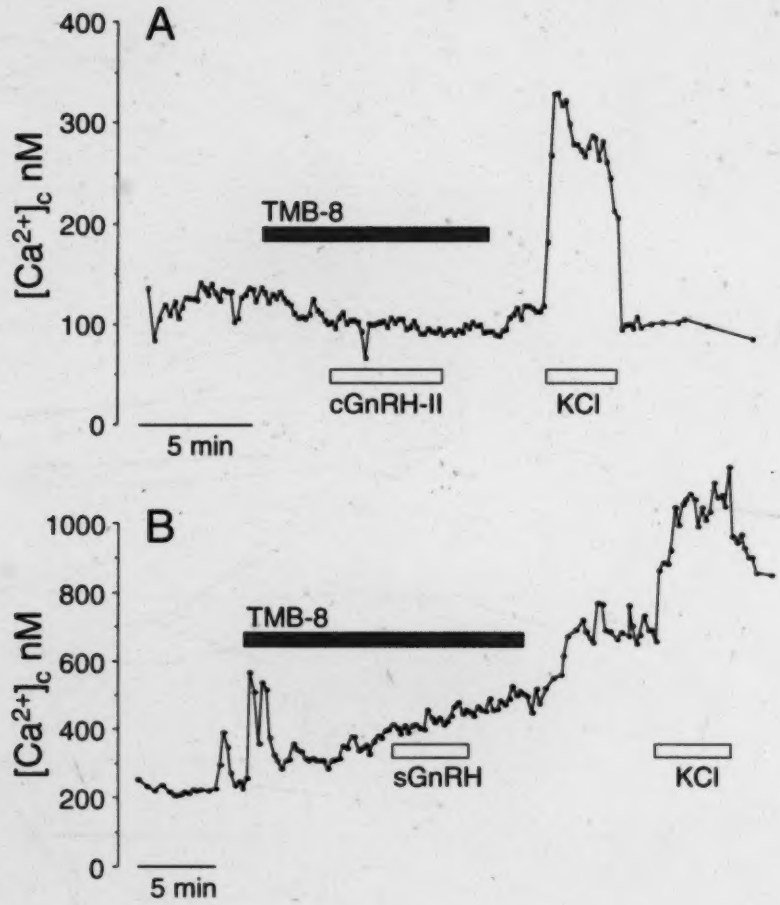

Figure 5.2. Intracellular Ca^{2+} stores are the source of GnRH-evoked Ca^{2+} signals. A) Treatment with 100 μM TMB-8 completely abolishes 100 nM cGnRH-II-stimulated Ca^{2+} signals. B) TMB-8 also blocks 100 nM sGnRH-evoked Ca^{2+} signals. As a positive control, 5 minutes of 30 mM KCl was added to activate VGCCs. Traces are representative of 8 cells (4 sGnRH, 4 cGnRH-II). In 3 of these cells, TMB-8 alone generated an increase in $[Ca^{2+}]_c$. All three cells responding to TMB-8 had pretreatment $[Ca^{2+}]_c$ slightly over 200 nM (spontaneously active), as compared to the other cells which had resting $[Ca^{2+}]_c$ under 150 nM.

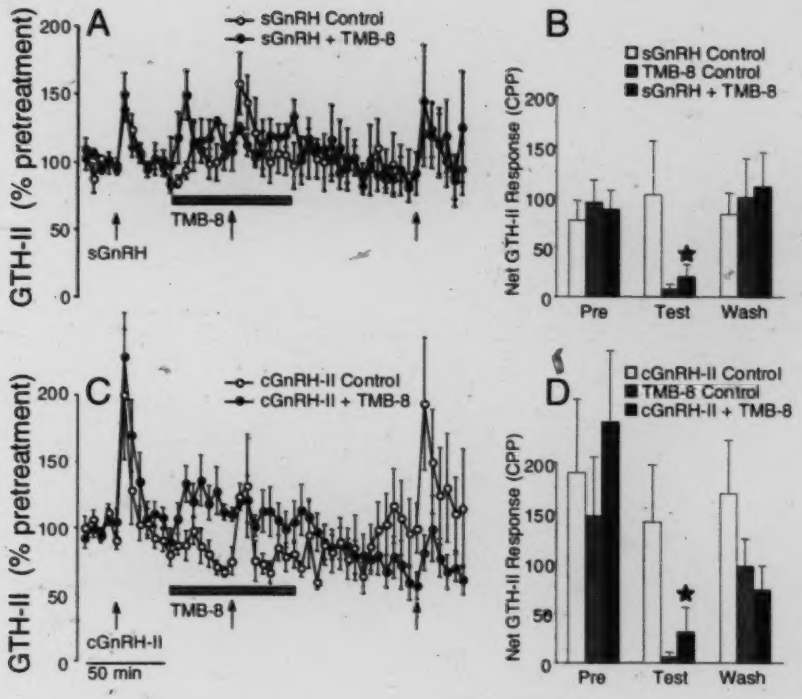

Figure 5.3. Intracellular Ca^{2+} stores mediate GnRH-stimulated GTH-II release. TMB-8 (*horizontal bar*) abolishes GTH-II release stimulated by100 nM sGnRH (A) or 100 nM cGnRH-II (C) in cell-column perifusion experiments compared to both parallel and sequential controls (n=6). One set of columns received 3 sequential GnRH pulses (GnRH control). A second set of columns (drug control) received a GnRH pulse (*pre*), then TMB-8 alone during the test period, followed by a GnRH pulse (*wash*). In a third set of columns (GnRH+TMB-8), the middle GnRH pulse (*test*) was given in the presence of TMB-8. *Arrows* denote 5-minute application of GnRH. Net responses to pretreatment, test and washout pulses of sGnRH (B) and cGnRH-II (D) are shown. The units are cumulative percent pretreatment (CPP) as in all other hormone release quantification graphs. Quantified GTH-II responses to test pulses (*black bars*) are significantly lower (*star*) than control GnRH pulses (*white bars*) and not different from the hormone release responses, quantified during the same time period, in columns exposed to TMB-8 alone during the test period (*gray bars*). For clarity, hormone release profiles in which GnRH was not given during TMB-8 treatment are not shown.

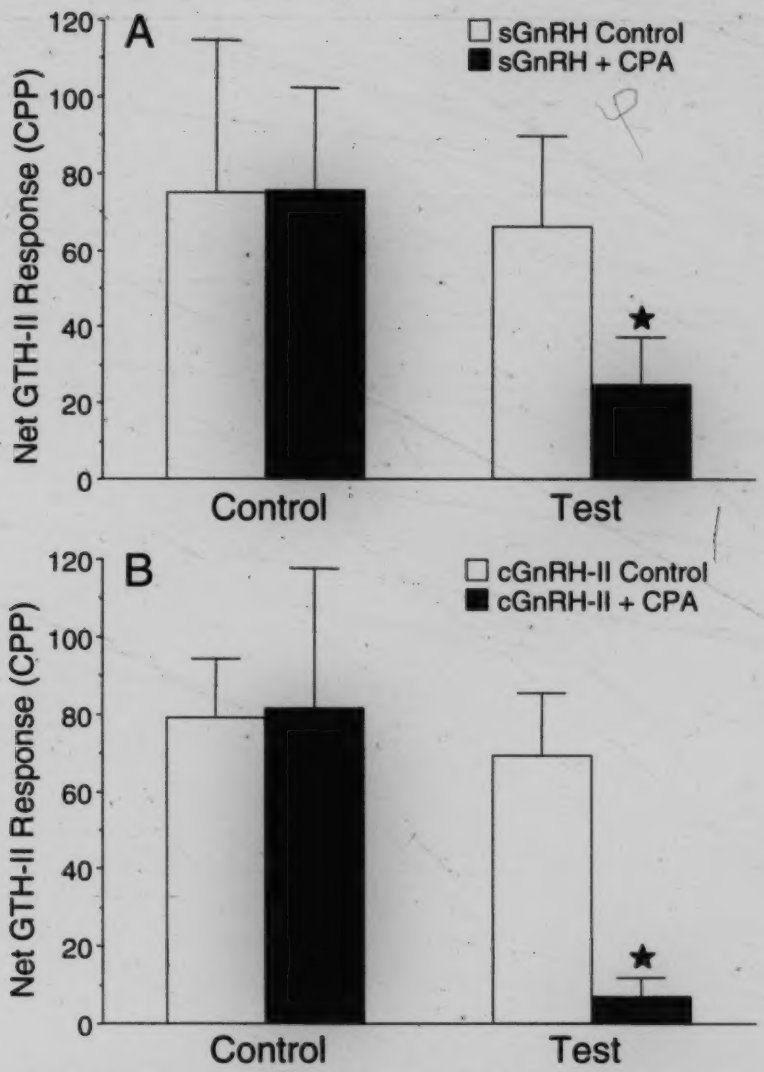

Figure 5.4. GnRH-stimulated GTH-II release requires SERCA pumps. Treatment with 10 μM CPA (30 minute pretreatment) inhibits GTH-II release stimulated by 100 nM sGnRH (A) or 100 nM cGnRH-II (B). Net response was quantified from parallel/sequentially controlled perifusion experiments, with the same design as those shown in Figure 5.3. Treatment with CPA alone did not significantly alter basal GTH-II secretion during the test period (not shown). Responses to both sGnRH and cGnRH-II recovered in washout pulses, which were not quantified because they were only used in 4 of 6 columns. *Star* indicates significant difference from control (n = 6).

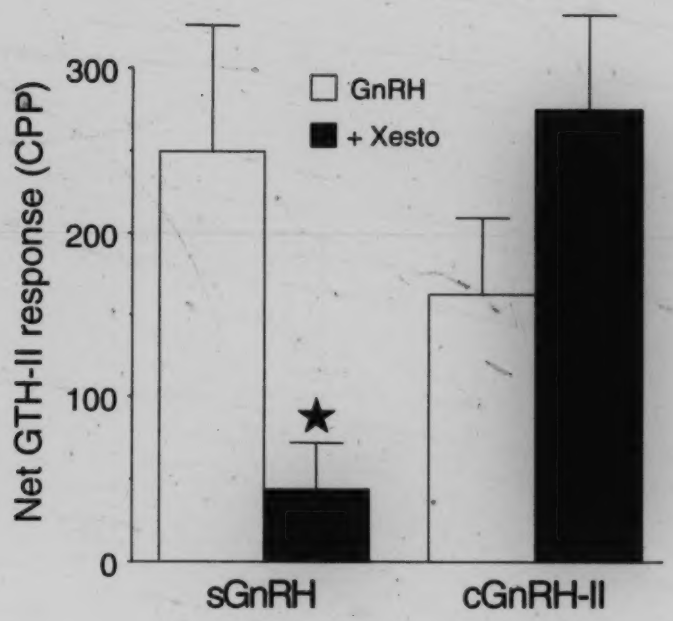

Figure 5.5. Differential involvement of IP_3 receptors in sGnRH- and cGnRH-II-stimulated GTH-II release. Treatment with xestospongin C (1 μM; 10-minute pretreatment) blocked 100 nM sGnRH-induced, but not 100 nM cGnRH-II-stimulated, GTH-II release response (quantified over 15 minutes) in perifusion experiments (1-minute fractions). *Star* indicates significant difference between quantified GnRH responses in the presence of xestospongin C and control GnRH pulses in parallel columns (*t*-test; n=4). Applications of sGnRH or cGnRH-II were given in parallel column and can therefore be directly compared. Sequential GnRH controls were not employed in these experiments.

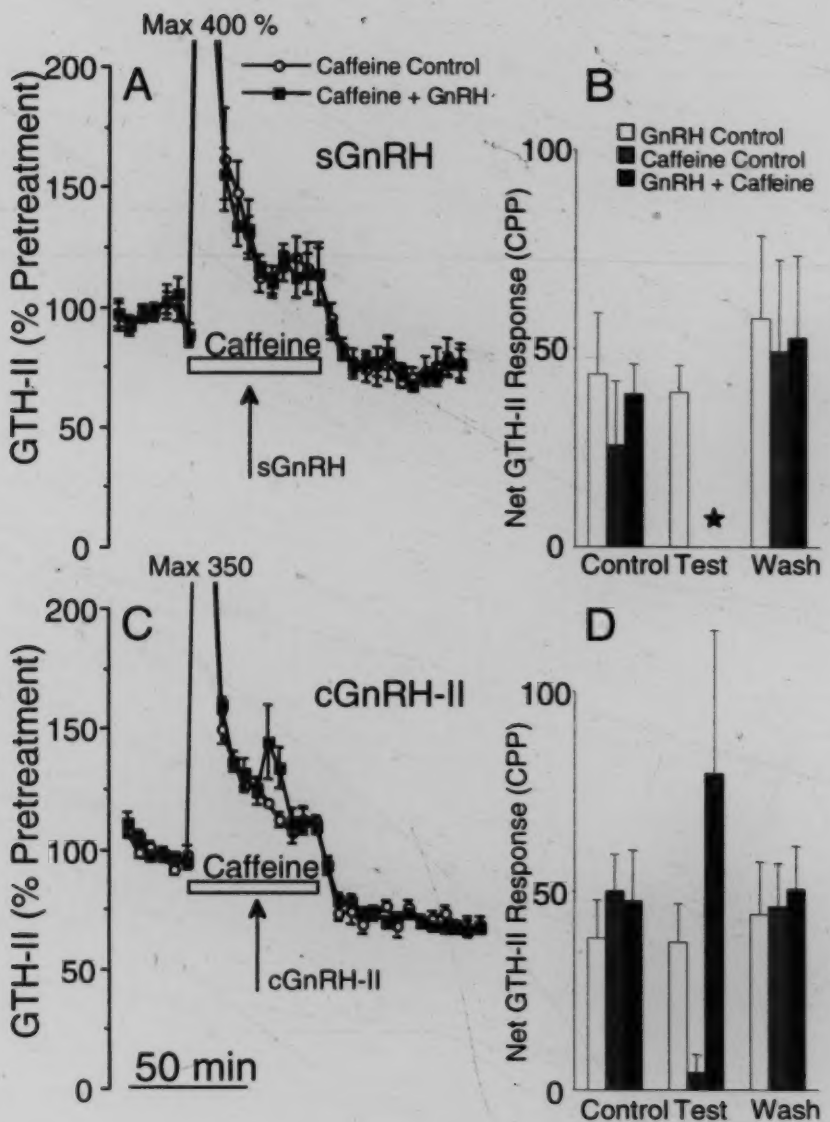
Max 400 %
A
Caffeine Control
Caffeine + GnRH
sGnRH
GTH-II (% Pretreatment)
200
150
100
50
0
Caffeine
sGnRH
B
GnRH Control
Caffeine Control
GnRH + Caffeine
Net GTH-II Response (CPP)
100
50
0
Control Test Wash
Max 350
C
cGnRH-II
Caffeine
cGnRH-II
50 min
D

Figure 5.6. Differential involvement of caffeine-sensitive intracellular Ca^{2+} stores in GTH-II release evoked by sGnRH and cGnRH-II. Caffeine (10 mM) alone evoked massive GTH-II release. A,C) Hormone release traces circumscribing the caffeine treatment period (*open horizontal bar*) and the 100 nM GnRH test pulse (*arrow*). Columns are designated using the same nomenclature as in Fig. 5.3. The response to sGnRH was completely abolished in the presence of caffeine (A). In contrast, the response to cGnRH-II was unaffected in the presence of caffeine (C). B and D) Quantified GTH-II responses from the complete experiment are shown. Hormone release profiles of pooled GnRH control columns (no caffeine), as well as pretreatment and washout pulses from other columns were omitted for clarity. Note that 2 columns in which cGnRH-II-stimulated GTH-II response was very large in the presence of caffeine were excluded from the time-course graph, but are included in the quantification. *Star* indicates significant difference from control (sGnRH n = 4, cGnRH-II n = 6).

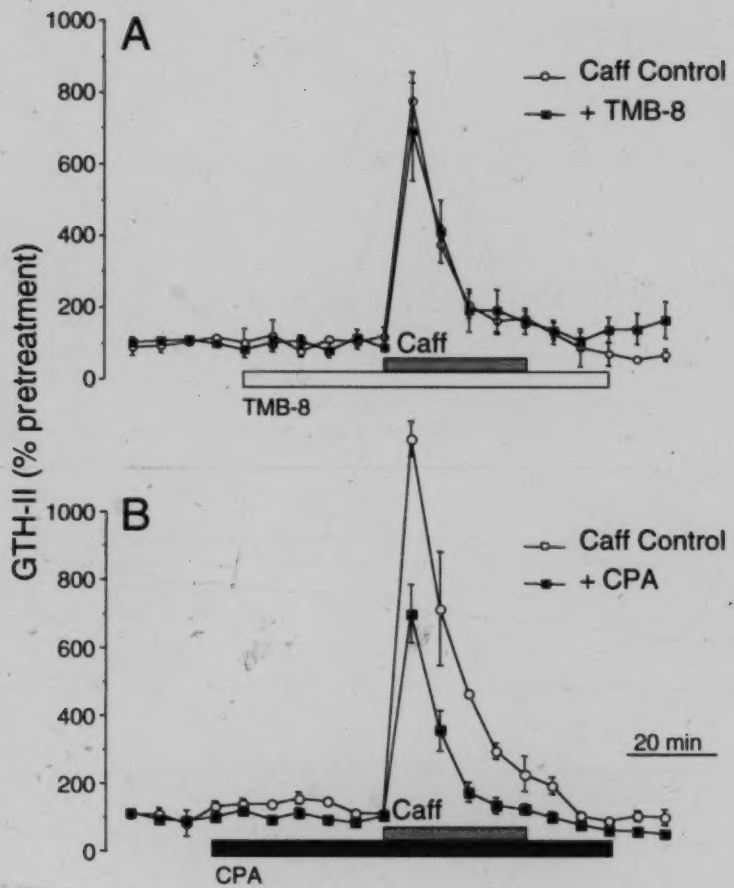

Figure 5.7. Relative functional independence of caffeine-sensitive Ca^{2+} stores and TMB-8/CPA-sensitive Ca^{2+} stores. A) Similarity of the 10 mM caffeine-stimulated GTH-II release (*gray bars*) in the presence or absence of 100 μM TMB-8 (*white bar*; n = 4). B) Comparison of caffeine-stimulated GTH-II release in the presence or absence of 10 μM CPA (*black bar*; n = 4).

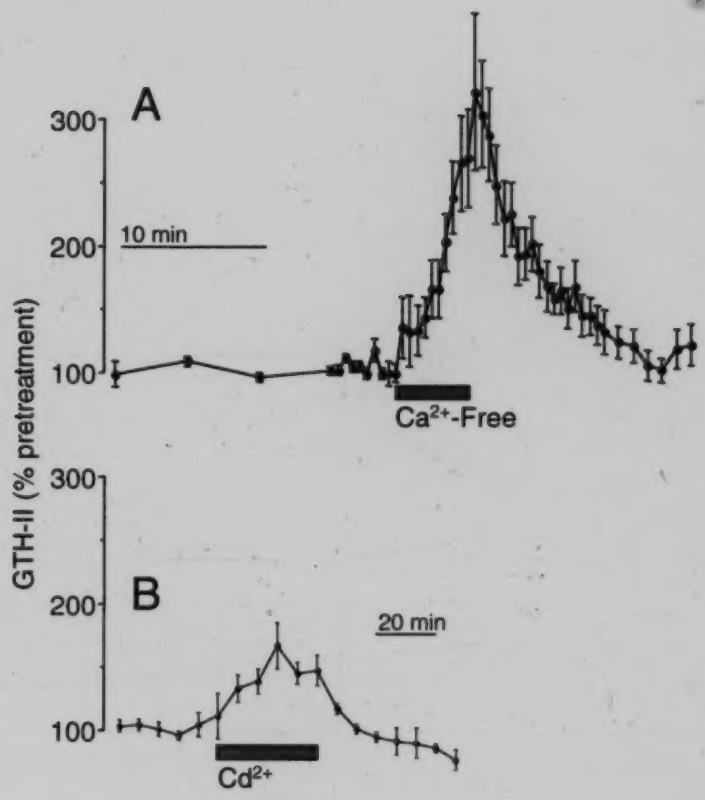

Figure 5.8. Paradoxical increase in GTH-II release during treatments which reduced the availability of extracellular Ca^{2+}. A) Application of nominally Ca^{2+}-free media (*gray bar*) leads to a rapid and robust GTH-II response in cell-column perifusion experiments (n = 12). B) Addition of 50 μM $CdCl_2$ (*gray bar*) also stimulates GTH-II secretion (n = 12).

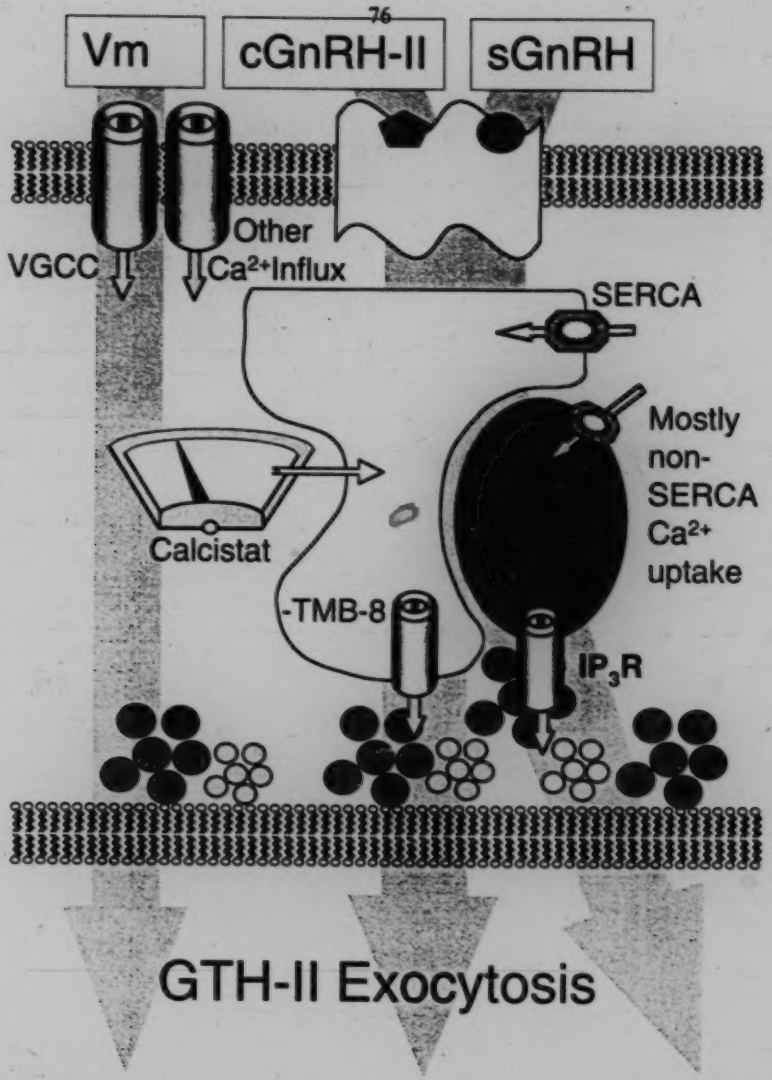

Figure 5.9. Working model of Ca^{2+} signaling systems leading to acute GTH-II release in goldfish gonadotropes. Shaded arrows represent simplified signaling pathways. One signaling pathway is linked to Ca^{2+} influx through VGCCs. Two separate GnRH signaling pathways emanate from the GnRH receptor. Both pathways involve a common intracellular Ca^{2+} store that is sensitive to TMB-8 and CPA/BHQ. Downstream, sGnRH-signaling bifurcates to include a distinct Ca^{2+} pool(s) sensitive to caffeine and/or xestospongin C. A putative homeostatic Ca^{2+} sensing mechanism (termed the calcistat), which is linked to GTH-II exocytosis, and possible intracellular Ca^{2+} stores, is also shown. Other plasma membrane Ca^{2+} fluxes may also be important.

Chapter 6.

Caffeine stimulated GTH-II release involves Ca^{2+} stores with novel properties

We have shown that 10 mM caffeine evokes robust secretion of GTH-II from cultured goldfish gonadotropes and that caffeine-sensitive Ca^{2+} stores mediate the GTH-II releasing effects of sGnRH, but not cGnRH-II (Chapter 5). Likewise, sGnRH, but not cGnRH-II, mobilizes a depletion-resistant intracellular Ca^{2+} store, which is likely sensitive to IP_3 (Jobin et al., 1992; reviewed in Chang et al., 1996; Chapter 5). The physiological relevance of these differences in signal transduction is underscored by the demonstration that these two closely related endogenous neuropeptides can generate quantitatively distinct Ca^{2+} signals in identified gonadotropes (Johnson et al., 1999) and differentially regulate GTH-II gene expression (Khakoo et al., 1994). Interestingly, over the course of the yearly reproductive cycle, the efficacy of caffeine-stimulated GTH-II release parallels that of sGnRH (see Chapter 8). Can caffeine be used as a probe for the selective study of sGnRH Ca^{2+} signalling in goldfish gonadotropes? Before this question can be answered, a deeper understanding of the mechanisms of caffeine-stimulated GTH-II release must be ascertained.

In this chapter, the properties of caffeine-induced GTH-II release and Ca^{2+} signals in goldfish gonadotropes were examined. Results demonstrated that caffeine-evoked GTH-II release was resistant to blockade of extracellular Ca^{2+} entry through VGCC, but completely dependant on caffeine's ability to generate Ca^{2+} signals. Unlike in other cells types, caffeine-stimulated hormone release was independent of the modulation of RyR or Ca^{2+}ATPases of the SERCA family. The characteristics of caffeine-stimulated GTH-II and the features of caffeine-evoked Ca^{2+} signals suggest that caffeine may mobilize Ca^{2+} from novel, localized stores in or around the secretory granules.

Results

Caffeine-stimulated GTH-II release is Ca^{2+} dependent

Perifusion of dispersed goldfish pituitary cells with 10 mM caffeine resulted in a very large GTH-II release response (Fig. 6.1A). A second application of caffeine (45 minutes after the first) evoked only the plateau portion of the GTH-II release response (Fig. 6.1B). The lack of a large 'peak' of GTH-II release indicates that some component may have become depleted or desensitized before the second caffeine application. Furthermore, it indicates that the 'peak' and the 'plateau' phases of caffeine-stimulate GTH-II release may be mediated by partially distinct mechanisms. It is important to note that it is during the plateau component when caffeine blocks sGnRH-stimulated GTH-II release. We have quantified both components separately (in addition to total response) in subsequent experiments.

In accordance with the idea that caffeine acts by mobilizing intracellular Ca^{2+} stores, caffeine generated Ca^{2+} signals in single identified gonadotropes (Fig. 6.2A,B). The GTH-II response to caffeine was abolished by loading cells with the high-affinity Ca^{2+} chelator BAPTA (accomplished by pre-incubation

with 50 µM BAPTA-AM; Fig. 6.2C), suggesting that caffeine-stimulated GTH-II release is not mediated by a parallel Ca^{2+}-independent signalling cascade. Although caffeine-elicited GTH-II responses were many fold greater than those induced by GnRH, Ca^{2+} signals evoked by caffeine were of similar maximal amplitude to those stimulated by sGnRH or cGnRH-II (Johnson et al., 1999; Chapter 4). Peak $[Ca^{2+}]_c$, measured over the bulk cytosol, rose only 157 ± 33 nM above baseline in the presence of 10 mM caffeine (n=6). It is important to note that the actual $[Ca^{2+}]_c$ in some regions of the cell is likely misrepresented by these bulk measurements since the peak of the caffeine-evoked Ca^{2+} signal was highly localized (Fig. 6.2B). In all cells tested, caffeine generated Ca^{2+} signals that were initially focused in the region opposite the nucleus, which is known to be rich in secretory granules (Van Goor et al., 1994), but subsequently spread across the cell. Collectively, these data suggest that caffeine-stimulated GTH-II release requires Ca^{2+} signals initiated by spatially localized Ca^{2+} stores.

VGCC do not mediate caffeine-stimulated GTH-II release

We have hypothesized a role for VGCC in the refilling of the rapidly-exchanging Ca^{2+} pools sensitive to both GnRHs. Goldfish gonadotropes possess VGCCs, which are high-voltage activated, inactivation resistant and can be completely blocked by micromolar Cd^{2+} (Van Goor et al., 1996). Blockade of VGCCs with simultaneous application of 50 µM Cd^{2+} did not block either phase of the caffeine-evoked GTH-II release response (Fig. 6.3). In separate experiments, we have shown that Cd^{2+} had no effect even when given up to 35 minutes before the application of caffeine (CJH Wong, JD Johnson and JP Chang, unpublished data), suggesting that caffeine-sensitive Ca^{2+} stores are not refilled through the activity of VGCCs. Treatment with 50 µM Cd^{2+} alone caused an increase in GTH-II release of variable amplitude (Fig. 6.3), an effect we have attributed to the homeostatic release of Ca^{2+} from intracellular stores (see Chapter 5).

Independence from typical RyR and SERCA-containing Ca^{2+} stores of the ER

The effects of 10 mM caffeine are most commonly attributed to the activation of RyR. In the present study, we used a high dose of ryanodine and a two-pulse protocol to test if caffeine-stimulated GTH-II release involves RyR. This protocol was selected because ryanodine binds to the RyR in its open conformation (Rousseau et al., 1987; Ehrlich et al., 1994) and, therefore, may exert a block that is use-dependent in some systems. Our results clearly demonstrated that 100 µM ryanodine had no effect on either caffeine pulse, compared to untreated controls (Fig. 6.4A,B). These negative results cannot be explained by a lack of functional ryanodine receptors in gonadotropes, since data described in Chapter 8 demonstrate that ryanodine can modulate hormone release in an manner consistent with its known mechanism of action and that RyR are involved in GnRH-stimulated GTH-II release. The GTH-II response evoked by caffeine was also not inhibited by 50 µM dantrolene, another inhibitor of RyR (Fig. 6.4C). At this time, we do not have a good explanation for the potentiation of caffeine-evoked GTH-II release by dantrolene. Collectively, these data indicate

that caffeine does not activate the RyR, which is presumed to be a component of ER Ca^{2+} stores (Berridge, 1997; Pozzan et al., 1994). Another hallmark of typical agonist-sensitive ER Ca^{2+} stores is the activity of SERCA pumps, which refill the components of the ER in all cell types studied to date (Pozzan et al., 1994). Treatment with 10 μM BHQ, a concentration that blocks SERCA (Mason et al., 1991) and that inhibits the Ca^{2+} stores common to both GnRHs in goldfish gonadotropes (Chapter 5), had no effect on caffeine-stimulated GTH-II release (Fig. 6.5). Results of experiments with structurally unrelated SERCA inhibitors show the same trend. Although CPA reduces caffeine-stimulated GTH-II release (Chapter 5), GTH-II release responses to caffeine are also completely unaffected by 2 μM thapsigargin (Chapter 11). Collectively, these findings suggest, albiet indirectly through the pharmacology of evoked hormone release, that caffeine-sensitive Ca^{2+} stores are largely independent of ER Ca^{2+}ATPases in goldfish gonadotropes.

Discussion

Relationship between caffeine- and sGnRH-sensitive Ca^{2+} stores

Caffeine-sensitive Ca^{2+} stores are highly coupled to GTH-II release in cultured goldfish gonadotropes. These Ca^{2+} stores are also of particular interest because they represent a clear difference between the signalling cascades of the major endogenous GTH-II secretagogues, sGnRH and cGnRH-II. Although acute GTH-II release stimulated by both GnRHs depends on the mobilization of intracellular Ca^{2+} stores which are sensitive to TMB-8 and SERCA inhibitors, only sGnRH uses an additional downstream caffeine-sensitive Ca^{2+} store (Chapter 5). This correlated well with the observation that GTH-II release evoked by sGnRH, but not cGnRH-II, requires an IP_3-sensitive Ca^{2+} store which is resistant to depletion under conditions that reduced the availability of extracellular Ca^{2+} (Jobin and Chang, 1992; Chang et al., 1996). The Ca^{2+}-dependence and the Cd^{2+}-insensitivity of caffeine-stimulated GTH-II release revealed in the present study, provide further evidence that caffeine may act on the same depletion-resistant Ca^{2+} store as sGnRH. The observation that GTH-II release responses in the presence of caffeine are greater than those of sGnRH-stimulated cells suggests the possibility that caffeine effectively mobilizes the majority of the Ca^{2+} in this pool(s), whereas sGnRH only releases a minor component of the store(s). Another possibility is that caffeine interacts with multiple intracellular Ca^{2+} stores or other signalling components.

Features of caffeine-evoked Ca^{2+} signals

Evidence from the present study, particularly those involving BAPTA, suggests that the ability to generate Ca^{2+} signals is essential to caffeine-stimulated GTH-II exocytosis. Additional evidence for a specific reliance on Ca^{2+} signalling is also provided by previous results which demonstrate that caffeine-stimulated GTH-II release is independent of cAMP/PKA pathways and K^+ channels. Specifically, caffeine-stimulated GTH-II release is completely unaffected by H89, a drug known to inhibit PKA with specificity in goldfish gonadotropes (CJH Wong, JP Chang, unpublished results; reviewed in Chang et al., 2000). Caffeine-

evoked GTH-II release is insensitive to TEA and 4-AP, effectively ruling out the possibility that caffeine acts by modulating K^+ channels (CJH Wong, JP Chang, unpublished results).

Given the disparity in the magnitude of the relatively small global Ca^{2+} signals and the large hormone release response, we speculate that caffeine evokes highly localized Ca^{2+} release from intracellular stores that are in close proximity to sites of GTH-II secretion. Consistent with this hypothesis, caffeine generated Ca^{2+} signals that began as highly localized events near a region of the membrane opposite the nucleus. Previous morphological studies have suggested that goldfish gonadotropes exhibit a weak polarity in culture and that this region is often rich in secretory granules and globules (Van Goor et al., 1994). The location of these events was similar in all cells tested, at least with respect to the ultrastructural landmarks (nucleus and globules) that are visible using DIC optics. One major advantage of our morphological identification protocol is that cells are selected for a specific orientation, thereby ensuring that they are imaged on roughly the same focal plane relative to the nucleus. However, beyond rough estimates of the localization of Ca^{2+} signals, the low spatial and temporal resolution of our data do not permit extrapolation to the level of Ca^{2+} microdomains, which are known to be important in the regulation of exocytosis and other localized cellular functions, in many cell types (for reviews see, Neher, 1998; Berridge, 1997; Johnson and Chang, 2000a).

Novel properties of caffeine-mediated Ca^{2+} release leading to exocytosis

In rat gonadotropes, Tse and colleagues (1997) have suggested that the strong coupling of mGnRH signalling to GTH-II exocytosis results from the close proximity of agonist-sensitive ER stacks to secretory granules (and presumably exocytotic protein machinery). In the rat model, acute mGnRH signalling follows a classical pathway and is mediated by a single Ca^{2+} pool that is IP_3-sensitive, rapidly-exchanging and refilled by SERCA pumps (Tse et al., 1993; Stojilkovic et al., 1994). Caffeine-sensitive Ca^{2+} stores, if present, have not been assigned a significant role in the generation of basal or GnRH-evoked Ca^{2+} signals in mammalian gonadotropes. However, a large body of work has demonstrated many differences in GnRH signal transduction and, specifically, Ca^{2+} signalling between rats and goldfish (Johnson et al., 1999; reviewed in Chang et al., 1996; Chang et al., 2000; Chapter 2). Indeed, the characteristics of caffeine-stimulated GTH-II release in the present study differ substantially from what we would expect of typical agonist-sensitive ER Ca^{2+} pools. First, caffeine-sensitive Ca^{2+} stores do not appear to be rapidly exchanging. Second, caffeine action is independent of RyR and SERCA pumps, which are known to mediate Ca^{2+} flux from caffeine-sensitive ER of many cell types (Pozzan et al., 1994). These features implicate several classes of non-ER Ca^{2+} stores, including acidic compartments such as the secretory granules, in sGnRH/caffeine-stimulated GTH-II release.

Given the localization of caffeine-evoked Ca^{2+} signals and the pharmacological evidence suggesting a role for non-ER Ca^{2+} stores, we speculate that caffeine may act on targets close to, or on the secretory granules themselves.

Secretory granules contain very large quantities of Ca^{2+}. In several cell types, Ca^{2+} accumulation into granules is SERCA independent; instead they are filled by H^+/Ca^{2+} exchange or possibly as a result of endocytosis (Martinez et al., 1996; Nicaise et al., 1992). The slow Ca^{2+} uptake was a primary reason why acidic organelles were rejected as candidates for the prototypical agonist-sensitive store, subsequently attributed to the ER (reviewed in Pozzan et al., 1994). Nevertheless, there is growing evidence that the secretory granules may constitute a physiologically-relevant IP_3 (agonist)-sensitive Ca^{2+} store, in a number of experimental systems (Nicaise et al., 1992; see Chapter 1). Although the most unequivocal evidence has come from studies of exocrine cell types (Nguyen et al., 1998; Gerasimenko et al., 1996a; Martinez et al., 1996), there is also evidence of novel Ca^{2+} stores associated with the secretory granules in endocrine cells (Blondel et al., 1996; Jonas et al., 1997). Gonadotropes have at least two distinct populations of secretory granules, based on ultrastructural studies (Nagahama, 1973; Kaul and Vollrath, 1974). It is possible that caffeine may activate highly-localized Ca^{2+} release from one of these granule populations which, due to its immediate proximity to exocytotic machinery and the plasma membrane, would result in a very large GTH-II release response.

Another related possibility is that sGnRH and caffeine may act on large acidophilic globules, which are independent of both populations of secretory granules and unique to gonadotropes in the goldfish (Nagahama, 1973; Kaul and Vollrath, 1974). Although these structures have been recognized at the electron microscope level for many years and their visibility under DIC optics is a major component of the morphological identification of goldfish gonadotropes (Van Goor et al., 1994), no function has yet been assigned to them. Interestingly, the apparent features of caffeine-sensitive stores in gonadotropes are quite different from the known properties of caffeine-sensitive Ca^{2+} stores in goldfish somatotropes (Chapter 7), which do not possess such structures. In the latter cell type, both GnRHs require caffeine-sensitive intracellular Ca^{2+} stores that are rapidly exchanging and blocked by TMB-8 (Johnson and Chang, 2000b; Chapter 7). This speculative model provides the basis for future investigations into the possibility that caffeine-sensitive intracellular Ca^{2+} stores with unique pharmacological and ultrastructural characteristics are involved in GTH-II exocytosis from goldfish gonadotropes. Future work, including the direct measurement of Ca^{2+} levels inside the secretory granules of intact goldfish gonadotropes, will be required to confirm our proposal.

Summary and physiological relevance

In the present report, we have characterized some pharmacological properties of an agonist-specific Ca^{2+} store relevant to the control of hormone release. It is tempting to speculate that caffeine-sensitive Ca^{2+} stores may be involved in the differential control of gonadotrope function by sGnRH and cGnRH-II (Khakoo et al., 1994). Caffeine and sGnRH have similar seasonal profiles, with respect to their maximum efficacy in releasing GTH-II *in vitro*, both being most efficient in between the stages of gonadal recrudescence and maturation (Lo and Chang, 1998; see Chapter 8). Compared to the kinetics of

cGnRH-II-stimulated GTH-II release, responses to sGnRH become very extended during this period (Lo and Chang, 1998), raising the possibility that the prolonged component may be due to the recruitment of slowly-exchanging caffeine-sensitive intracellular Ca^{2+} stores. Ca^{2+} signals generated by sGnRH also have slower rates of rise and more often exhibit a prolonged monophasic waveform, when compared to those of cGnRH-II (Johnson et al., 1999; Chapter 4). Although it has been elegantly demonstrated in a few cell types (e.g. Dolmetsch et al., 1998; reviewed in Johnson and Chang, 2000a), the functional specificity of Ca^{2+} signals has not been demonstrated within single pituitary cells, from any species.

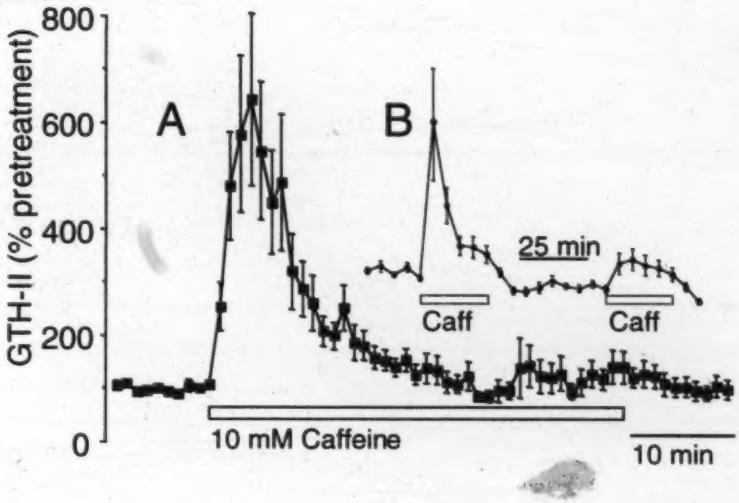

Figure 6.1. Temporal characteristics of caffeine-stimulated GTH-II release. A) GTH-II release response evoked by 10 mM caffeine (*white bars* through figure) are measured using 1-minute fractions (n=6). B) Caffeine-stimulated GTH-II release responses desensitize (n=8). Amplitude is scaled as in A.

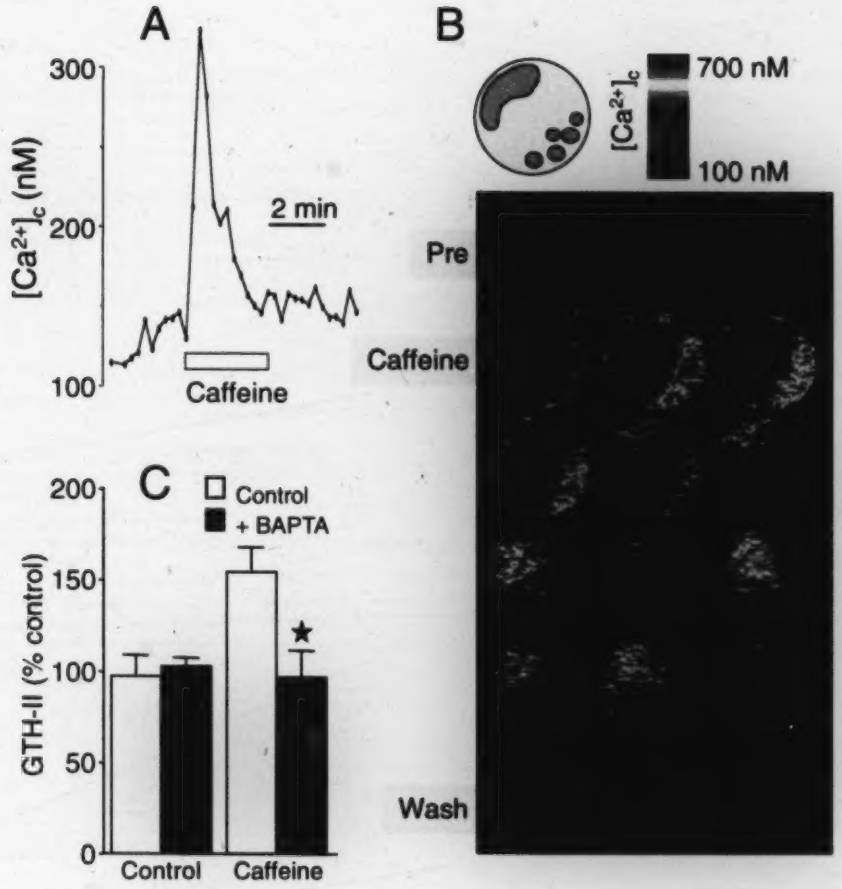

Figure 6.2. Localized caffeine-evoked Ca^{2+} signals mediate caffeine-stimulated GTH-II release. A) 10 mM caffeine (*white bar*) elevates $[Ca^{2+}]_c$ in identified gonadotropes (n=6). B) Pseudo colour representation of the spatial features of caffeine-evoked Ca^{2+} signals. Images are 15 seconds apart and are read from left to right, starting at the top. A cartoon of this gonadotrope is provided for reference. Example is representative of data obtained from the same cells as A. C) Pre-incubation of cells with 50 µM BAPTA-AM abolishes caffeine-stimulated GTH-II release in static incubation experiments (n=20).

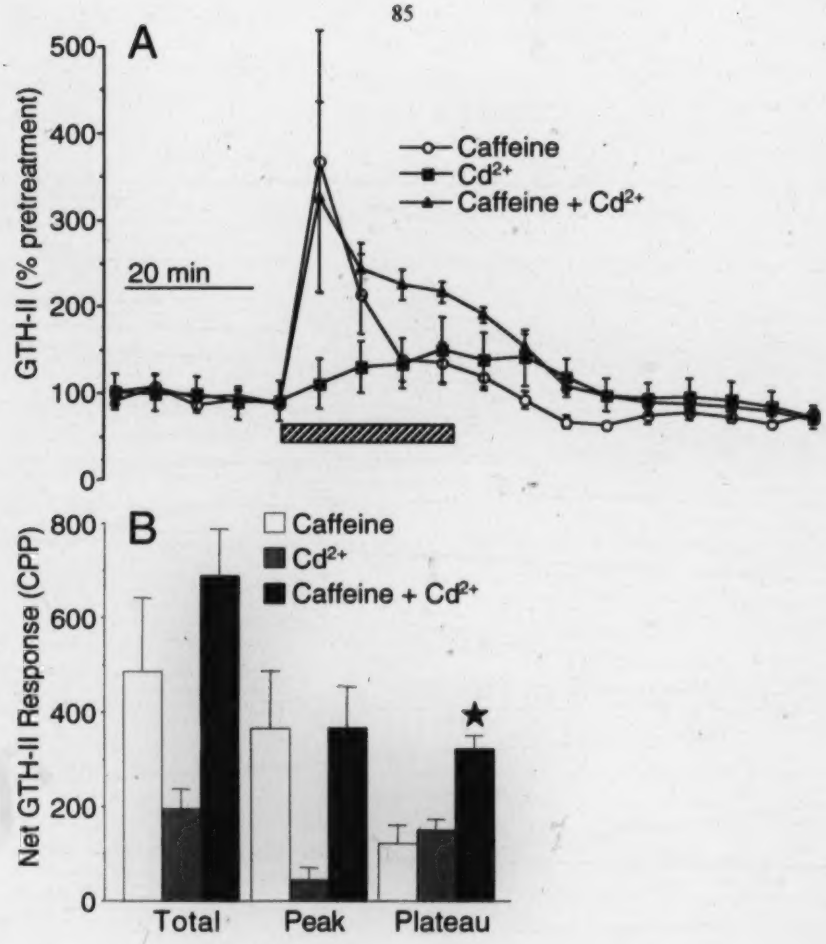

Figure 6.3. Caffeine-stimulated GTH-II release is not inhibited by VGCC blockade. A) GTH-II release response to10 mM caffeine is not inhibited by simultaneous application of 50 μM Cd^{2+} (*hashed bar*). B) Quantified data from A. *Star* indicates significant difference from the response to caffeine alone.

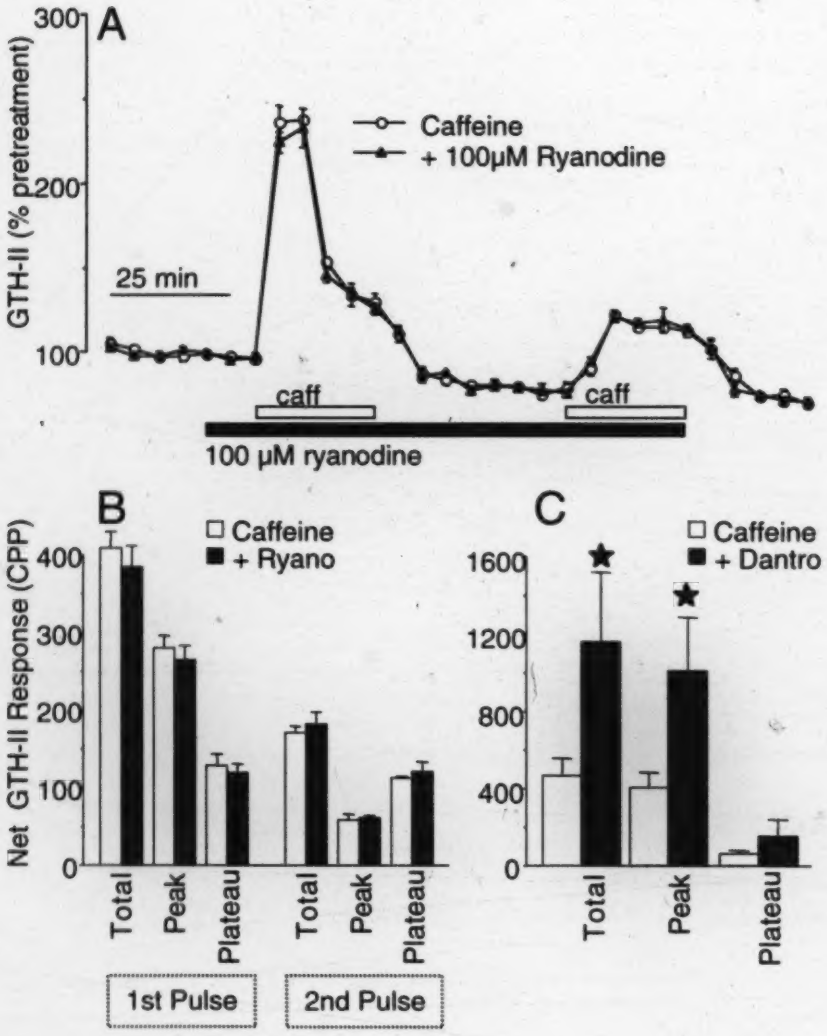

Figure 6.4. Caffeine-stimulated GTH-II release does not require RyR. A) Treatment with 100 μM ryanodine (*black bars*) does not inhibit sequential GTH-II responses elicited by caffeine (*white bars*). B) Data from Part A are quantified (n = 8). C) Caffeine-stimulated GTH-II release is not inhibited by 50 μM dantrolene (30 minute pretreatment) in perifusion experiments similar in design to those shown in Figure 6.5. (n = 8).

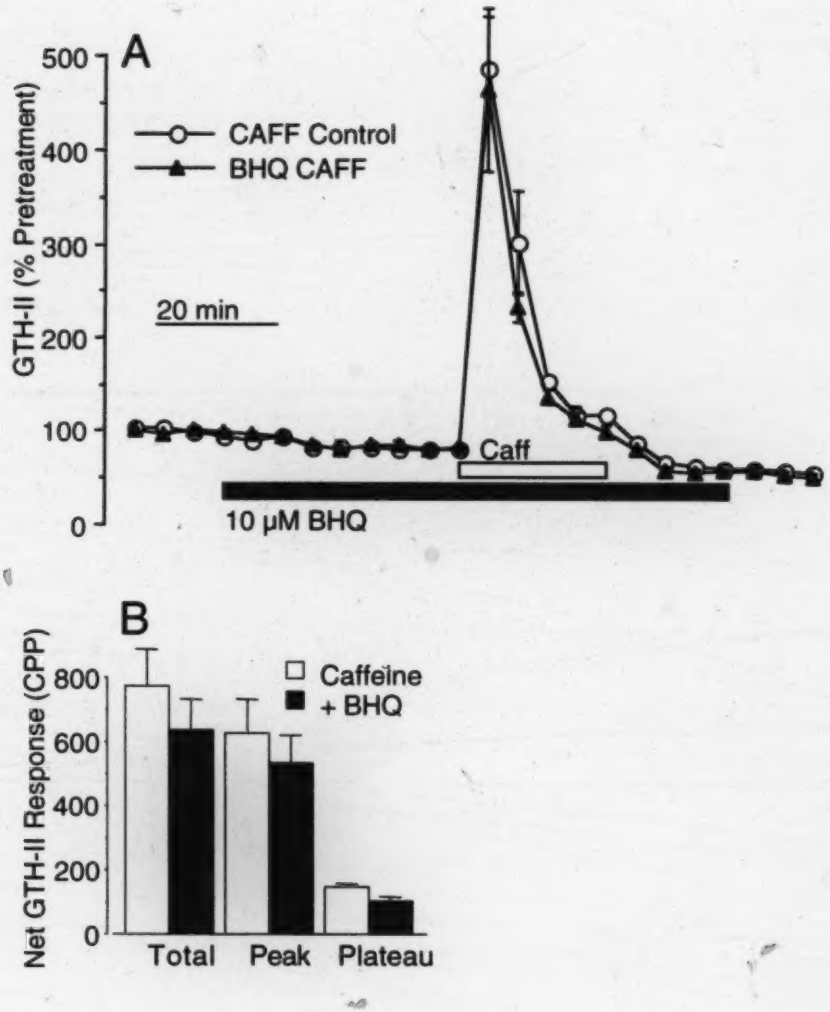

Figure 6.5. A) Caffeine-stimulated GTH-II release does not require SERCA Ca^{2+} ATPases. Effects of 10 µM BHQ (*black bar*) on GTH-II release evoked by 10 mM caffeine (*white bar*). B) displays quantified GTH-II responses (n = 8).

Chapter 7.

Novel, thapsigargin-insensitive intracellular Ca^{2+} stores control growth hormone release from goldfish pituitary cells*

The long-term maintenance of agonist-evoked goldfish GH release is dependent on the availability of extracellular Ca^{2+} and is attenuated by prolonged incubation with VGCC inhibitors (reviewed in Chang et al., 1996; Chang et al., 2000). Although goldfish somatotropes possess VGCC (Chang et al., 1994), there is no direct electrophysiological evidence for their involvement in acute agonist-stimulated GH release. Previous pharmacological studies have suggested a prominent role for VGCC-mediated Ca^{2+} entry in GnRH-stimulated GH release. However, these studies employed protocols where Ca^{2+}-free media or blockers of extracellular Ca^{2+} influx were applied far in advance (often >40 minutes) of GnRH treatment, which would likely deplete intracellular Ca^{2+} stores (Van Goor et al., 1999; Tse et al., 1993; see Chapter 5). Thus, previous experiments on the goldfish somatotrope model were unable to distinguish whether VGCC activation initiates the Ca^{2+} signal leading to secretion or is secondary to release of Ca^{2+} from intracellular stores. In the later scheme, the role of VGCC-mediated Ca^{2+} influx would be to replenish the intracellular Ca^{2+} stores.

The primary aim of this chapter was to determine the involvement of intracellular Ca^{2+} stores in GnRH-evoked GH release from goldfish pituitary cells *in vitro* using treatments that directly modulate intracellular Ca^{2+} release. The secondary goal was to characterize the pharmacological profile of agonist-sensitive Ca^{2+} pools in these cells. Results indicate that acute GnRH-stimulated GH release from goldfish somatotropes requires intracellular Ca^{2+} stores sensitive to TMB-8 and caffeine, but not to thapsigargin.

Results

A role for $[Ca^{2+}]_c$ in sGnRH and cGnRH-II signalling was supported by Fura-2 Ca^{2+} imaging of single identified goldfish somatotropes. Both neuropeptides elicit Ca^{2+} signals in naive cells (Fig. 7.1A,B,C; Fig. 7.2) which were 152 ± 25 nM and 88 ± 28 nM above baseline, for sGnRH and cGnRH-II, respectively. This is the first demonstration of agonist-induced $[Ca^{2+}]_c$ increases in identified somatotropes in lower vertebrates and provides novel evidence that sGnRH and cGnRH-II can act on the same cell (Fig. 7.1C). Ca^{2+} signals in the present study are quantitatively and qualitatively similar to GnRH-stimulated Ca^{2+} signals in goldfish gonadotropes (Johnson et al., 1999; Chapter 4). As in the latter cell type, the Ca^{2+} signals in somatotropes can be grouped by temporal response profiles as biphasic, monophasic or oscillatory (data not shown). In contrast to that previous study, fewer GnRH-evoked Ca^{2+} signals were highly localized with respect to the basal $[Ca^{2+}]_c$ gradients. On the other hand, GnRH-evoked Ca^{2+}

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signals were spatially distinct from those generated by VGCC activation with 30 mM KCl (Fig. 7.2) in somatotropes.

Next we used BAPTA to directly assess the involvement of Ca^{2+} signals on basal and GnRH-stimulated GH release. In this study, in addition to abolishing both sGnRH- and cGnRH-II-evoked GH release, pre-incubating cells with 50 μM BAPTA-AM also reduced basal GH secretion (Fig. 7.1D). These data support the well-accepted hypothesis that elevations in $[Ca^{2+}]_c$ are required for regulated exocytosis. Furthermore, the later result indicates that Ca^{2+} signalling is required to sustain maximal basal secretion in this cell type.

To further examine if the onset of GnRH-stimulated GH release is sensitive to manipulation of extracellular Ca^{2+} availability, GH secretion was measured at higher time resolution (30-second fractions). In these experiments, control cells and cells exposed to nominally Ca^{2+}-free media secreted GH at identical rates for the first 2 minutes of the GnRH challenge, indicating that extracellular Ca^{2+} availability is not crucial for the initiation of the GH response to GnRH (Fig. 7.3). After approximately 2 minutes, an extracellular Ca^{2+}-dependent component is manifested, suggesting that replenishment of somatotrope Ca^{2+} stores with extracellular Ca^{2+} is required to sustain GnRH-stimulated GH release. Interestingly, after some delay, the Ca^{2+}-free medium alone evoked a small increase in hormone secretion which could account for a portion of the GH release response that appears dependent on extracellular Ca^{2+}. We suspect that this may be a compensatory release of Ca^{2+} from intracellular stores (see below).

The relative involvement of Ca^{2+} signals generated by VGCC and intracellular Ca^{2+} mobilization in acute GnRH-stimulated GH release was further examined. Addition of a depolarizing concentration of KCl (30 mM) increased GH secretion (Fig. 7.4A,B) consistent with the presence of L-type VGCC in goldfish somatotropes (Chang et al., 1994). GnRH-evoked GH responses were unaffected during KCl treatment, which would be expected to activate VGCCs without depleting intracellular Ca^{2+} stores (Fig. 7.4A,B).

Next we examined the role of caffeine-sensitive Ca^{2+} stores in GnRH-stimulated GH release. Caffeine directly activates Ca^{2+} release from intracellular Ca^{2+} stores by sensitizing Ca^{2+}-release channels to existing levels of Ca^{2+} (Pozzan et al., 1994) and has been extensively used in many mammalian and non-mammalian cell types (e.g. Kostyuk and Kirischuk, 1993). 10 mM caffeine stimulated robust GH secretion (Figs. 7.4C,D). In the continuous presence of caffeine, pulses of sGnRH or cGnRH-II failed to elicit additional GH release (Figs. 7.4C,D), suggesting that GnRH-stimulated GH secretion is mediated by intracellular Ca^{2+} stores. Although additional experiments are required, it is unlikely that this block arises from a depletion of releasable hormone, since sequential caffeine treatments evoke similar levels of secretion (Fig. 7.8B).

Having revealed a role for intracellular Ca^{2+} stores in GnRH signalling in goldfish somatotropes, we conducted experiments with TMB-8, a broad-spectrum inhibitor of Ca^{2+} release from intracellular stores that has been used extensively in a variety of cell models, including goldfish cells (Van der Kraak, 1991; Malgodi and Chiou, 1974; Chini and Dousa, 1996; Anderson et al., 1992). GnRH-induced

Ca^{2+} signals and GH secretion were abolished in the presence of 100 μM TMB-8 (Figs. 7.5, 7.6). The effects of TMB-8 on hormone secretion were partially reversible after 75 minutes of washout in columns where a test pulse of GnRH had been added, and fully reversible in TMB-8 control columns (when compared to control responses from the same column; Fig. 7.5 insets). Further experiments with two sequential GnRH pulses during the washout period confirmed that the recovery of GnRH-stimulated GH release after TMB-8 treatment is both time- and use-dependent (n=4; data not shown). Although TMB-8 blocked both sGnRH- and cGnRH-II-stimulated Ca^{2+} transients, subsequent application of KCl could still generate robust Ca^{2+} transients (Fig. 7.6). These KCl-stimulated GH responses were seen during a time-period in which hormone release from goldfish somatotropes would normally be refractory to a subsequent GnRH challenge (see above). These data indicate that an independent pathway for Ca^{2+} signalling is still active in TMB-8-treated cells. Taken together, these results reinforce the hypothesis that the signalling pathways that mediate the acute secretagogue actions of GnRH on goldfish somatotropes require Ca^{2+} release from TMB-8-sensitive intracellular stores and is relatively independent of VGCC activation.

Many cells possess multiple intracellular Ca^{2+} stores. The pharmacology of the intracellular Ca^{2+} stores linked to GnRH-stimulated GH release was further investigated using Tg, which is a potent and selective inhibitor of all known isoforms of SERCA (Lytton et al., 1991). The molecular target of Tg includes the IP_3-sensitive Ca^{2+} stores of the endoplasmic reticulum in most cell types studied, including rat somatotrope-derived cell lines (Pizzo et al., 1997; Pozzan et al., 1994). Although Tg-sensitive Ca^{2+} stores participate in Ca^{2+} homeostasis in goldfish somatotropes (2 μM Tg increases $[Ca^{2+}]_c$ of goldfish somatotropes in both normal and Ca^{2+}-free media, see Chapter 12), sGnRH- and cGnRH-II-stimulated GH release was not inhibited by 2 μM Tg (25 minute pretreatment; Fig. 7.7). Similarly, sGnRH- and cGnRH-II-evoked GH responses were not blocked by two other structurally unrelated SERCA inhibitors, 10 μM CPA and 10 μM BHQ (n=6 for both, data not shown). Taken together, these results provide pharmacological evidence that SERCA do not refill the intracellular Ca^{2+} stores that mediate acute GnRH-stimulated GH release.

The features of caffeine-induced GH release were further examined to gain insight into the properties of GnRH-sensitive Ca^{2+} stores (Fig. 7.8). Caffeine-stimulated GH release did not desensitize when given 25 minutes apart, suggesting that the Ca^{2+} stores mediating these responses may be rapidly-exchanging (Fig. 7.8B). Preincubation with 50 μM BAPTA-AM, abolished caffeine-stimulated GH release, indicating a dependence on Ca^{2+} signals (Fig. 7.8C). As was the case with GnRH, acute caffeine-stimulated GH release was relatively insensitive to co-application of a treatment that reduces the availability of extracellular Ca^{2+} (50 μM Cd^{2+}; Fig. 7.8D). Note that the first five minutes of caffeine-stimulated hormone release were completely unaffected by Cd^{2+}. The pharmacological profile of caffeine-evoked GH release also mimicked that of GnRH with regard to intracellular Ca^{2+} antagonists. Caffeine-stimulated GH release was severely abrogated by 100 μM TMB-8, but was insensitive to 2 μM Tg (Fig. 7.9). In addition, CPA and BHQ exerted only partial inhibition on

the GH release response to caffeine (Fig. 7.10). When viewed together, these data strongly suggest that sGnRH, cGnRH-II and caffeine act on a common TMB-8-sensitive Ca^{2+} store, which is largely SERCA independent. Surprisingly, caffeine-stimulated GH release was not blocked by 100 μM Ry (Fig. 7.11). Collectively, these findings clearly suggest that GnRH- and caffeine-sensitive Ca^{2+} stores share many of the same functional properties in goldfish somatotropes. Moreover, these data suggest that multiple Ca^{2+} stores exist in goldfish somatotropes.

The involvement of intracellular Ca^{2+} stores in prolonged secretion was also tested. TMB-8 significantly reduced basal secretion in 2-hour static-incubation experiments (Fig. 7.12), indicating that the 'basal' hormone release activity blocked by BAPTA may arise from spontaneous release of Ca^{2+} from intracellular stores. Direct evidence for this was attained by imaging $[Ca^{2+}]_c$ in single somatotropes. Captured just before the application of TMB-8, the average basal $[Ca^{2+}]_c$ in the present study was 222 ± 41 nM. However, of all the 33 cells used in this study, 8 had resting Ca^{2+} levels over 200 nM and were spontaneously active (to varying degrees) with Ca^{2+} peaks of several hundred nanomolar in some cases. Without the spontaneously active cells included, the basal $[Ca^{2+}]_c$ was 110 ± 9 nM. These two groups of somatotropes behaved differently when treated with TMB-8. In the spontaneously active group, application of TMB-8 resulted in an immediate cessation of spiking, and in most cases a rapid drop in $[Ca^{2+}]_c$ to around 100 nM (10 cells; Fig. 7.6, top panel), suggesting that the fluctuating high $[Ca^{2+}]_c$ observed in these cells is due to the spontaneous release from TMB-8 sensitive Ca^{2+} stores. In cells with low basal $[Ca^{2+}]_c$ (~100 nM) TMB-8 did not reduce Ca^{2+}. Instead, Ca^{2+} levels remained stable or increased slightly (4 cells; Fig. 7.6, bottom panel). The occasional increases in $[Ca^{2+}]_c$ following TMB-8 application may reflect a compensatory flux of Ca^{2+} from other stores or from the extracellular media. The sensitivity of goldfish somatotropes to manipulations that alter Ca^{2+} availability is in agreement with the alterations to cellular Ca^{2+} homeostasis presumed to underlie the small increase in basal GH secretion seen in Ca^{2+}-free media (Fig. 7.3).

Consistent with their effects on acute agonist-stimulated GH release, neither sGnRH nor cGnRH-II stimulated GH release in the presence of TMB-8 or caffeine over 2-hours in static incubation (Fig. 7.12A,B). In contrast, TMB-8 had no effect on GH release evoked by 10 μM ionomycin (Fig. 7.12C). Thus, the TMB-8 block on intracellular Ca^{2+} release channels can be completely circumvented by ionophore-mediated Ca^{2+} translocation across the plasma membrane, as well as, intracellular membranes. The latter experiment demonstrates that TMB-8 actions on hormone release are not due to non-specific effects on Ca^{2+}-effector proteins. Taken together, these data demonstrate that intracellular Ca^{2+} stores are also important in prolonged GH release from goldfish somatotropes.

Finally, we used TMB-8 as a probe for the involvement of intracellular Ca^{2+} stores in two signal transduction pathways that are prominent in activation-secretion coupling in goldfish somatotropes (PKC and cAMP; reviewed in Chapter 2; Chang et al., 1996; Chang et al., 2000). Interestingly, TMB-8 affected forskolin-stimulated, but not phorbol ester-induced, GH release. This indicates

that TMB-8-sensitive Ca^{2+} stores are important in the long-term GH-releasing actions of the cAMP-dependent, but not the PKC-dependent, pathways (Fig. 7.12C). In order to evaluate whether VGCCs are active under basal conditions, we treated cells with 10 μM BayK 8644, which promotes an increase in probability of opening and the open time in active VGCCs, but does not open VGCCs in the absence of a depolarization (Kokubin and Reuter, 1984). Although BayK 8644 increases current through VGCCs in goldfish pituitary cells (Van Goor et al., 1996), this manipulation had no significant effect on GH release, suggesting increasing the open probability of VGCCs alone is insufficient to activate GH exocytosis.

Discussion

Results presented in this chapter demonstrate that intracellular Ca^{2+} stores mediate the acute actions of sGnRH and cGnRH-II on GH secretion in goldfish somatotropes. In addition, the GnRH-evoked Ca^{2+} signals and the pharmacology of these Ca^{2+} stores, were partially characterized. Ca^{2+} signals generated by physiological agonists have not been reported for identified somatotropes from non-mammalian vertebrates. GnRH-stimulated Ca^{2+} signals in goldfish somatotropes are similar in amplitude to those found in goldfish gonadotropes (Johnson et al., 1999; Chapter 4) and bovine somatotropes (Rawlings et al., 1991). On the other hand, Ca^{2+} signals generated by sGnRH or cGnRH-II were smaller than those evoked by KCl depolarization, as was found for rat somatotropes (Tomic et al., 1999b). It is interesting to note that both neuropeptides generate Ca^{2+} signals on the same identified somatotropes. Since many neuroendocrine regulators, including both GnRHs (reviewed in Peter and Chang, 1999), are physiologically relevant at the level of the pituitary, this result indicates that goldfish somatotropes are an excellent model for studies of interacting signal transduction pathways.

Our results also confirm the requirement for Ca^{2+} signals in goldfish GH release. Experiments where cells were pre-incubated with BAPTA-AM indicate that evoked hormone release responses are entirely dependent on GnRH-stimulated Ca^{2+} signals. Under these conditions, basal GH secretion was also significantly reduced. This indicates that a component of basal GH secretion involves the spontaneous release of Ca^{2+} from intracellular stores (Missiaen et al., 1991), since the reduction in hormone release is mimicked by TMB-8 (Fig. 7.12), but not consistently by the removal of extracellular Ca^{2+} (Wong et al., 1994). This prediction was supported by the observation of TMB-8-senstitive spontaneous Ca^{2+} signals in a subpopulation of somatotropes (e.g. Fig. 7.6 upper panel) and by experiments in which basal $[Ca^{2+}]_c$ is not reduced in Ca^{2+}-depleted media (JD Johnson, CJH Wong and JP Chang, unpublished). It should be noted that TMB-8 treatment only slighly reduced basal GH release in perifusion studies on naive cells (Fig. 7.9). The reason for this discrepency is unclear, although it may be related to the low temperature at which cells are perifused (18°C versus ~21°C for imaging and 28°C for static culture). Nonetheless, in contrast to the present study, spontaneous $[Ca^{2+}]_c$ oscillations in rat somatotropes are mediated by the influx of extracellular Ca^{2+} (Holl et al., 1988; Tomic et al., 1999b).

It is interesting to note that spontaneous Ca^{2+} signalling is generally not found in other cell types in the goldfish pituitary. When compared to simultaneously-imaged somatotropes, gonadotropes and unidentified cells have lower resting $[Ca^{2+}]_c$, exhibit much less spontaneous Ca^{2+} activity and do not show a reduction in basal $[Ca^{2+}]_c$ with TMB-8 treatment (see Chapter 5). Somatotropes also have comparatively higher $[Ca^{2+}]_c$ in non-perifused cells (n=15; data not shown). In accordance with the hypothesis that spontaneous elevations in $[Ca^{2+}]_c$ are responsible for the BAPTA- and TMB-8-sensitive components of basal GH release, basal GTH-II release from the same cultures was not inhibited by either agent (Chapter 5; JD Johnson and JP Chang, unpublished data). Taken together, these results indicate that the involvement of TMB-8 sensitive Ca^{2+} stores in spontaneous Ca^{2+} signalling and basal GH release may be cell-type specific among cultured goldfish pituitary cells.

The conclusions presented here are at variance with previous interpretations of the relative involvement of Ca^{2+} from intracellular and extracellular sources in GH release from teleost pituitary cells (reviewed in Chang et al., 1996). However, given the limitations of the previous studies, they must now be reinterpreted. Several lines of evidence suggest that VGCCs are not required for the initiation of GnRH-stimulated GH release in goldfish; 1) GnRH- and KCl- stimulated hormone release are additive, 2) GnRH and KCl evoke Ca^{2+} signals which are spatially distinct, 3) the initial 2 minutes of hormone secretion appear to be completely independent of extracellular Ca^{2+}, 4) BayK 8644 was insufficient in stimulating GH release over 2 hours and GnRH-evoked changes in membrane potential have not been recorded in goldfish pituitary cells, 5) BayK 8644 does not potentiate GH release evoked by the same concentration of GnRH used in the present study (Chang et al., 1994). Taken together these data strongly suggest that GH secretion is driven by rapidly exchanging intracellular Ca^{2+} stores that are highly sensitive to the manipulation of extracellular Ca^{2+} availability, such as those that mediated GnRH signalling in mammalian systems (Tse et al., 1993; Van Goor et al., 1999).

Most importantly, data presented in this chapter provide direct evidence for a dominant intracellular Ca^{2+} component in GnRH-stimulated GH release. Both TMB-8 and caffeine abolished acute GnRH-stimulated GH release, strongly suggesting that intracellular Ca^{2+} stores are required for GnRH signalling. The findings that prolonged incubations (2-hour static) with TMB-8 or caffeine also block GnRH-stimulated GH release, indicate that Ca^{2+} stores, when they have access to extracellular Ca^{2+}, probably control long-term secretion as well. The long-term involvement of intracellular Ca^{2+} stores in GH release is consistent with previous observations that significant agonist-evoked GH release is still evident in long-term treatments that reduce the availability of extracellular Ca^{2+}, such as VGCC inhibitors (Chang et al., 1994). The observation that caffeine evokes a massive secretory response indicates that Ca^{2+} release from intracellular stores is also sufficient to regulate GH release. Taken together with the observation that Tg did not alter acute basal GH secretion (see Fig. 7.9B and Chapter 12), these data indicate that GnRH-stimulated GH release, and part of basal GH release, is

dependent on Ca^{2+} signals generated by Tg-insensitive intracellular Ca^{2+} stores (Fig. 7.13).

The process by which Ca^{2+} stores of goldfish somatotropes are refilled is currently unknown. Since somatotropes contain dihydropyridine-sensitive VGCCs (Chang et al., 1994) and depolarization can lead to Ca^{2+} influx, we propose that VGCCs may play a role in the refilling process. Whether or not the eventual activation of VGCCs can be targeted by inhibitory or stimulatory neuroendocrine regulators remains to be elucidated. Some studies in goldfish somatotropes have suggested that PKC may play a role in enhancing VGCC activity (reviewed in Chang et al., 1996; Chang et al., 2000), although direct electrophysiological evidence is lacking. The results of the present study indicate that PKC involvement in GnRH signal transduction must be downstream or independent of the initial Ca^{2+} signal generated by intracellular Ca^{2+} stores. In addition, the involvement of VGCCs may be indirect, with intermediate compartments, such as the mitochondria (Babcock et al., 1997) aiding in the refilling of ER Ca^{2+} stores. Alternatively, the TMB-8/caffeine-sensitive Ca^{2+} stores may also be refilled by a store-operated Ca^{2+} current, as in other cell types (Bennett et al., 1998).

Multiple functionally and pharmacologically distinct non-mitochondrial Ca^{2+} stores are thought to coexist in many cell types, including normal and clonal rat somatotropes (Petit et al., 1999; Herrington and Hille, 1994; Tanaka and Tashjian, 1993). In GH_4C_1 cells, at least three intracellular Ca^{2+} pools can be functionally and pharmacologically distinguished (Tanaka and Tashjian, 1993). Imaging and pharmacological evidence from in intact astrocytes and myocytes demonstrates that distinct caffeine- and Tg-sensitive Ca^{2+} stores may co-exist in the ER (Golovina and Blaustein, 1997). Consistent with the work of Tanaka and Tashjian, (1993), the results of the present study are consistent with a model in which TMB-8/caffeine-sensitive Ca^{2+} stores are functionally independent of those modulated by Tg. In most systems, Tg and IP_3 are thought to target the same Ca^{2+} pool(s). The involvement of IP_3 signalling in GnRH-stimulated GH release is discussed in Chapter 8. Whether other neuroendocrine regulators use such IP_3/Tg-sensitive Ca^{2+} stores in goldfish somatotropes remains to be investigated.

In many systems, the caffeine-sensitive Ca^{2+} pool overlaps with the Ca^{2+} stores containing the ryanodine receptor Ca^{2+}-release channel. Although goldfish somatotropes possess functional ryanodine-sensitive Ca^{2+} stores, neither the GnRHs (Chapter 8) nor caffeine (Fig. 7.11) utilize these stores to evoke GH release, suggesting that the caffeine/TMB-8 stores in the present study posses a novel pharmacology.

Ironically, previous experiments designed to evalulate the role of VGCC in agonist-stimulated GH release may actually provide further clues regarding the identity of the GnRH-sensitive intracellular Ca^{2+} stores revealed in this study. Drugs such as nifedipine, verapamil, diltiazem and BayK 8644, at doses used to inhibit VGCCs, may actually modulate a recently-discovered class of intracellular Ca^{2+} stores! The unique intracellular Ca^{2+} release channels gated by the novel Ca^{2+}-mobilizing agent, NAADP, are sensitive to many pharmacological modulators of voltage-sensitive Ca^{2+} channels (Genazzani et al., 1996). It is

interesting that NAADP-stimulated intracellular Ca^{2+} mobilization is completely blocked by TMB-8 in sea urchin egg homogenates (Chini and Dousa, 1996). Recent studies have demonstrated that NAADP plays a role in the initiation of agonist Ca^{2+} signals in pancreatic acinar cells (Cancela et al., 1999). Future investigations are required to determine the involvement of this novel Ca^{2+} signalling system in GnRH-stimulated GH release and its interaction with other Ca^{2+} pools. It is clear that goldfish somatotropes possess multiple independent Ca^{2+} signalling pathways coupled to GH secretion, including one that is voltage-dependent. Thus, the goldfish somatotrope is an ideal model for the study of multiple, parallel Ca^{2+}-dependent signal transduction cascades.

In rat somatotropes, Ca^{2+} signalling pathways involving VGCCs have been assigned a paramount role in the generation of spontaneous Ca^{2+} events, as well as agonist-evoked GH release (Holl et al., 1988; Tomic et al., 1999b; reviewed in Chen et al., 1994). Notwithstanding, intracellular Ca^{2+} stores have been implicated in the signal transduction of endothelin and GHRP-6 (Tomic et al., 1999a; Herrington and Hille, 1994). More recently, GHRH signalling was also shown to require participation of intracellular Ca^{2+} stores in the rat (Petit, 1999). These findings suggest that mammalian somatotropes may possess multiple, parallel, agonist-specific Ca^{2+} signalling pathways leading to exocytosis as has been indicated in the present study. It is clear that an integrated model of multiple Ca^{2+} signalling pathways is required in both model systems.

In conclusion, we propose that a novel class of intracellular Ca^{2+} stores mediates acute and prolonged GnRH-stimulated GH release from cultured goldfish somatotropes. These Ca^{2+} stores are differentially involved in cAMP- and protein kinase C (PKC)-dependent signalling systems, which have been implicated in the signal transduction of dopamine and GnRH, respectively (Chang et al., 2000). The same caffeine/TMB-8-sensitive Ca^{2+} store is also responsible for the spontaneous Ca^{2+} release and GH secretion, which may be unique to a population of somatotropes. The involvement of VGCCs is likely distal to the activation of stores and serves to replenish this rapidly-depleting pool, through a mechanism that either does not involve SERCA pumps or involves Ca^{2+}-ATPases with novel pharmacology.

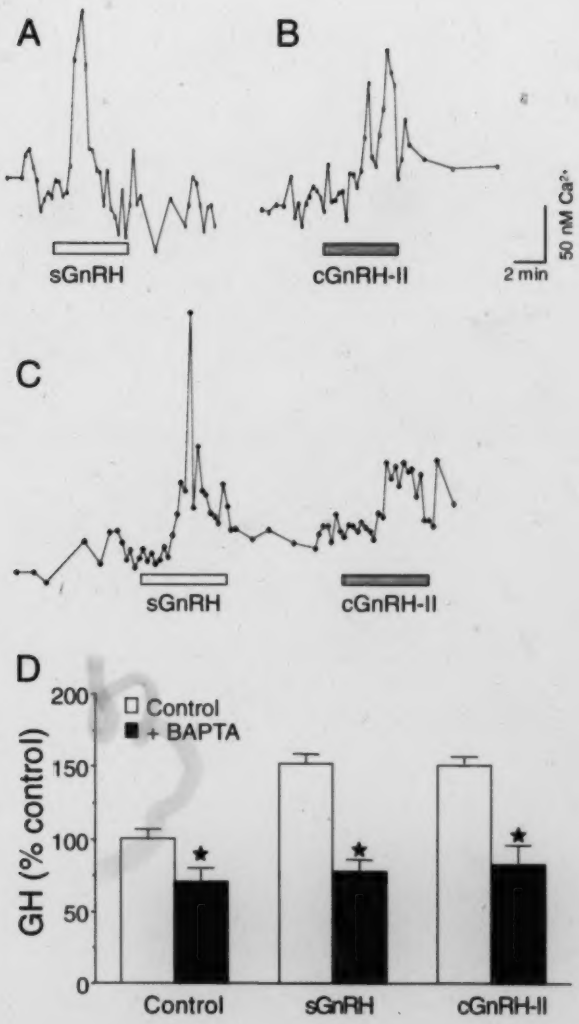

Figure 7.1. Involvement of Ca^{2+} signals in GnRH action in somatotropes. The temporal profiles of transient Ca^{2+} signals generated by 100 nM sGnRH (A) or 100 nM cGnRH-II (B) are shown. The maximal amplitude above the baseline is 152 ± 25 nM for sGnRH-evoked Ca^{2+} signals and 88 ± 27 nM for cGnRH-II-evoked Ca^{2+} signals. Both neuropeptides stimulated Ca^{2+} signals when administered to the same cell (C). Traces are representative of 8, 5 and 6 independent trials for panels A, B and C, respectively. The requirement for Ca^{2+} signals in basal and 100 nM sGnRH- or 100 nM cGnRH-II-stimulated GH release over 2 hours is shown by loading cells with 50 μM BAPTA-AM (D). *Stars* indicate significant difference from non-BAPTA controls (n=12).

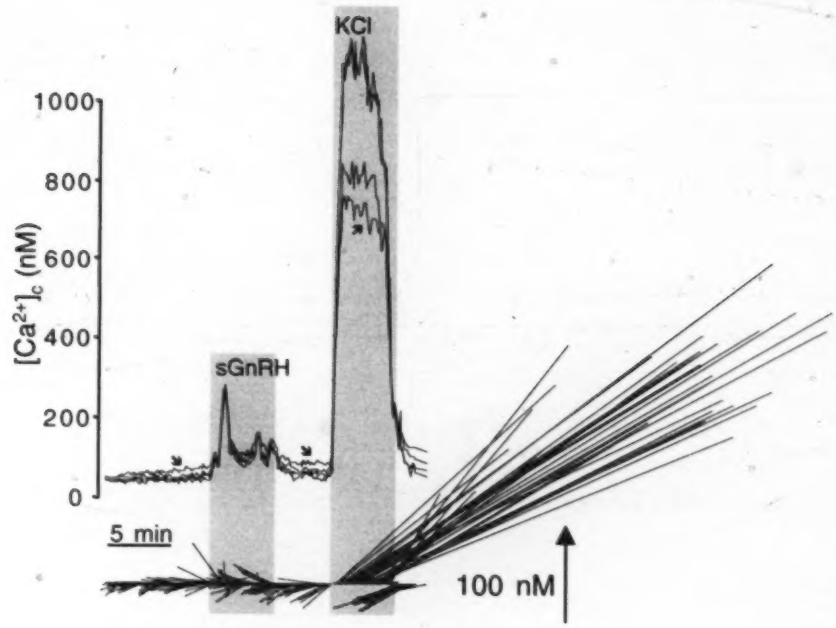

Figure 7.2. Differential spatial patterns of Ca^{2+} signals evoked by GnRH and depolarization. Ca^{2+} gradients are relatively stable in 100 nM sGnRH-II-stimulated somatotropes (similar results were seen with 100 nM cGnRH-II). In contrast, Ca^{2+} gradients are reversed in the presence of 30 mM KCl. Data are representative of 5 cells. This effect can also been seen by following the Ca^{2+} levels in a one quadrant (*small arrows*). *Large arrow* indicates a gradient of 100 nM in the nuclear direction.

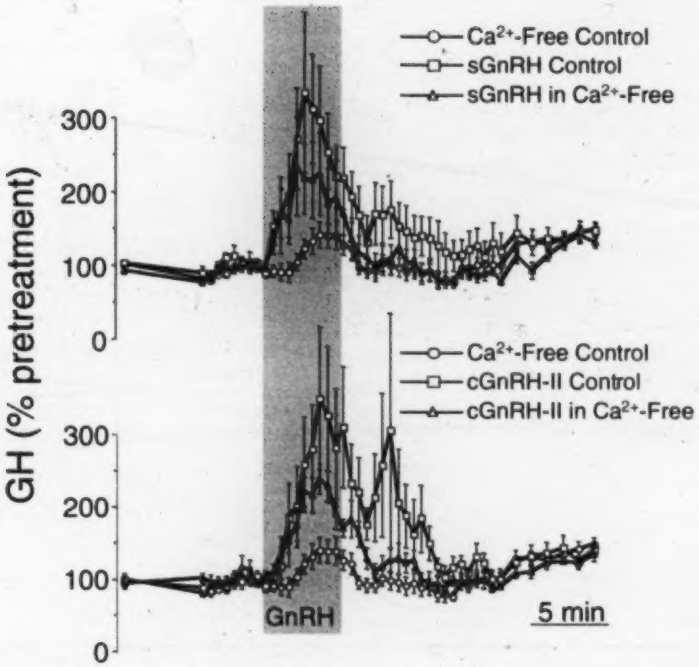

Figure 7.3. The initiation of GnRH-stimulated GH release is independent of the availability of extracellular Ca^{2+}. Dispersed goldfish pituitary cells were perifused with either 100 nM sGnRH (*top panel*) or 100 nM cGnRH-II (*bottom panel*) in the presence or absence of Ca^{2+}-depleted media (made without Ca^{2+} salts). Samples were collected every 30 seconds. (n=6)

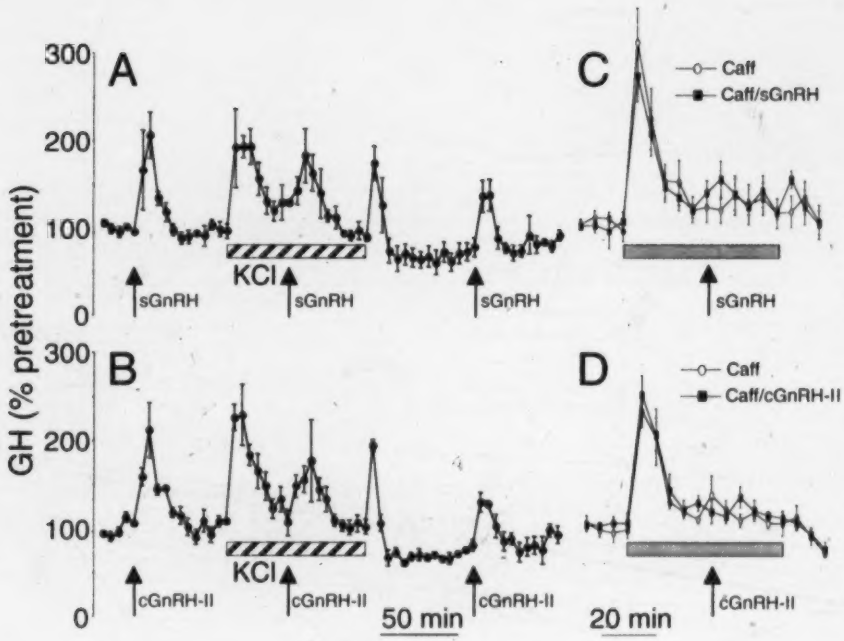

Figure 7.4. Comparison of the effects of VGCC activation and release of Ca^{2+} from caffeine-sensitive intracellular stores on basal and GnRH-stimulated GH release. Activation of VGCC with media containing 30 mM KCl (*hashed bars*) does not block subsequent responses to 5-minute application of either 100 nM sGnRH (A) or 100 nM cGnRH-II (B), as indicated by arrows. sGnRH- and cGnRH-II-evoked GH responses during KCl stimulation are not significantly different from control GnRH challenges (pretreatment or washout controls), when quantified as described in Chapter 3 (n=4). The rebound seen after KCl application has not been reliably observed in other similar experiments in our laboratory (JP Chang, unpublished). In separate experiments, neither 100 nM sGnRH (C) nor 100 nM cGnRH-II (D) stimulate GH release when intracellular Ca^{2+} stores have been depleted with 10 mM caffeine. Caffeine alone is also a potent secretagogue. (n=4).

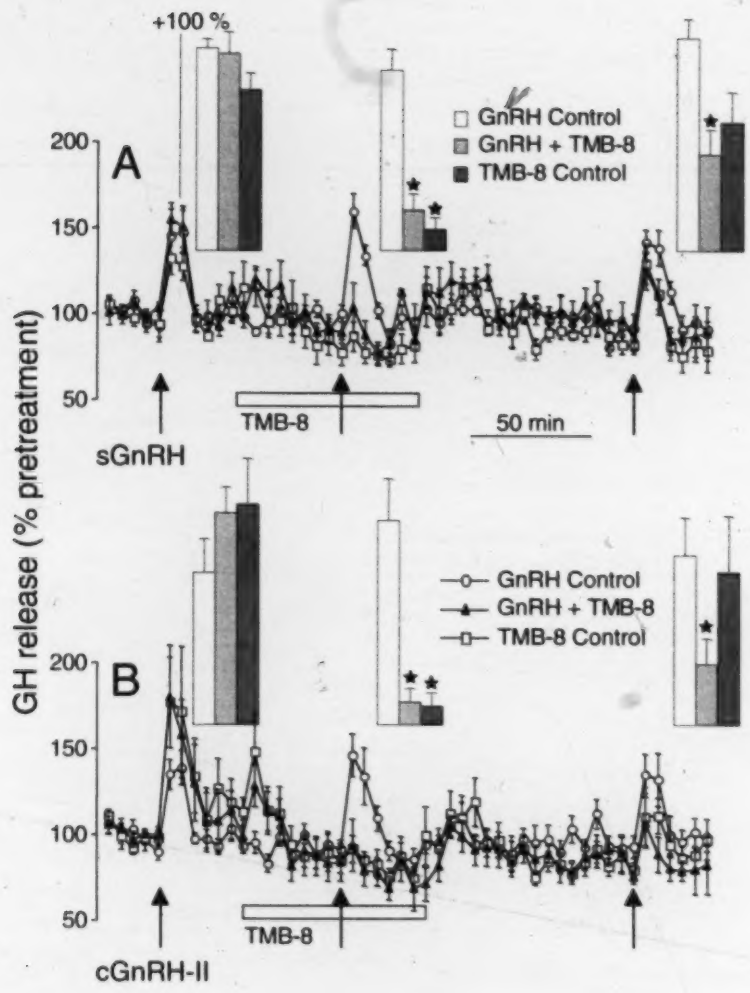

Figure 7.5. Acute GnRH-stimulated GH release is abolished by a broad-spectrum blocker of intracellular Ca^{2+} release channels. Treatment with 100 μM TMB-8 (*white bars*) blocks the GH response to 5-minute challenges of either 100 nM sGnRH (A) or 100 nM cGnRH (B) as indicated by *arrows*. *Insets* are net GH responses over their appropriate GnRH pulse quantified as CPP. (n=6). *Stars* indicate responses that are significantly different from the control pulse of that column (1st pulse, pretreatment).

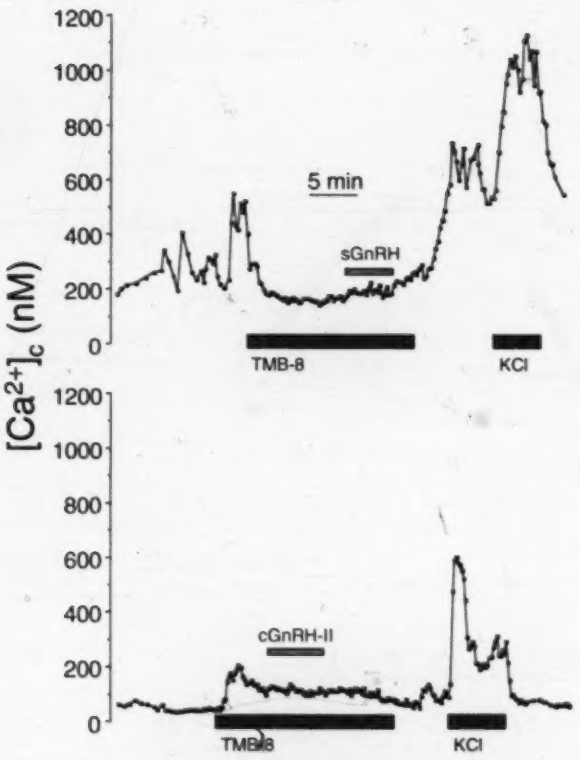

Figure 7.6. GnRH-evoked Ca^{2+} signals depend on Ca^{2+} release from TMB-8-sensitive intracellular stores. Representative traces of the effect of 100 μM TMB-8 on Ca^{2+} signal generation by 100 nM sGnRH (*bar in top panel*; n=9) or 100 nM cGnRH-II (*bar in bottom panel*; n=5) in identified somatotropes. The parallel, voltage-dependent Ca^{2+} signalling pathway, activated by 30 mM KCl, leading to GH release remains active in somatotropes that have been exposed to TMB-8. TMB-8 transiently decreased Ca^{2+} levels in spontaneously-active GH cells with high resting $[Ca^{2+}]_c$ (*top panel*), but not in some somatotropes with lower resting $[Ca^{2+}]_c$ (*bottom panel*).

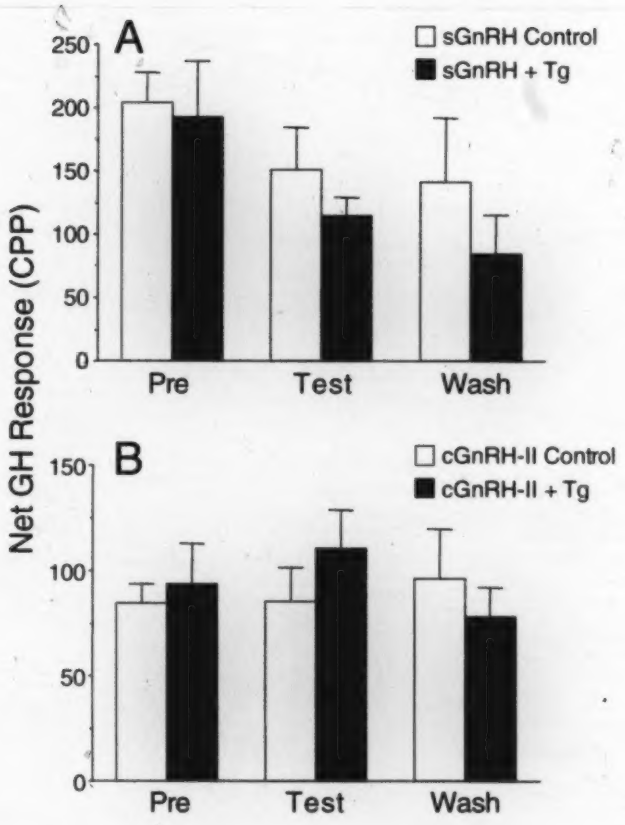

Figure 7.7. Thapsigargin-sensitive intracellular Ca^{2+} stores do not mediate acute GnRH-stimulated GH release from goldfish somatotropes. Shown are quantified GH responses from cell column perifusion experiments where the effects of 2 μM Tg on 100 nM sGnRH-stimulated (*top panel*) or 100 nM cGnRH-II-stimulated (*bottom panel*) GH release are examined. All responses are statistically similar (n=8).

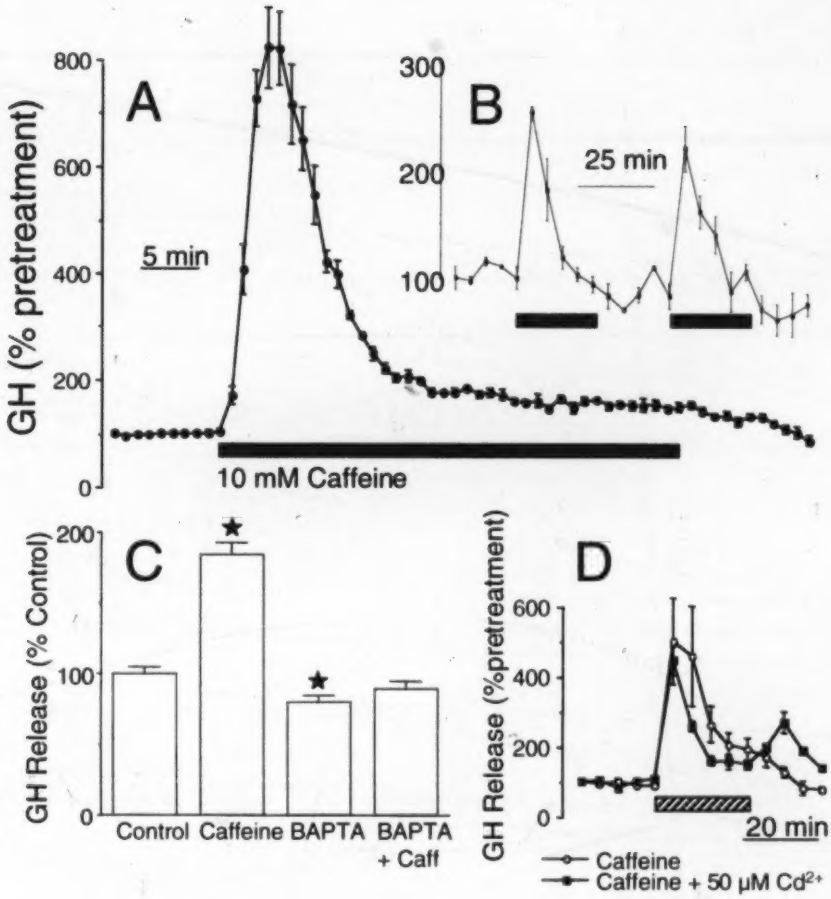

Figure 7.8. Characteristics of caffeine-stimulated GH release. A) Temporal profile of the GH release response to 10 mM caffeine (*black bar*; n=6). Samples were collected every 60 seconds. B) Caffeine-stimulated GH release does not desensitize over 25 minutes (n=8). C) Caffeine-stimulated GH release over 2 hours is abolished by pre-incubation in 50 µM BAPTA-AM (n=20). *Stars* indicate difference from untreated control. D) Simultaneous application of 50 µM Cd^{2+} (*hashed bar*) does not inhibit the peak phase (first 5 minutes) of 10 mM caffeine-evoked GH release (n=6).

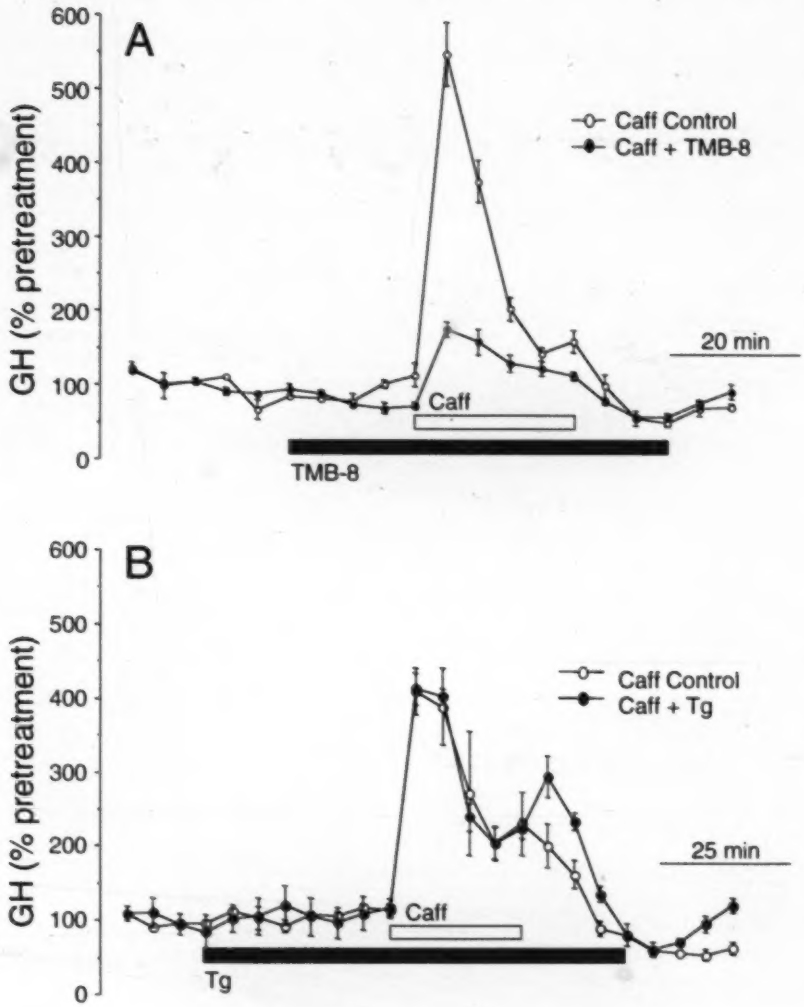

Figure 7.9. Caffeine-stimulated GH release is mediated by TMB-8-sensitive, but not Tg-sensitive, intracellular Ca^{2+} stores. A) Treatment with 100 μM TMB-8 dramatically reduces the GH response of 10 mM caffeine. B) In contrast, 2 μM Tg does not inhibit 10 mM caffeine-stimulated GH release. (n=4).

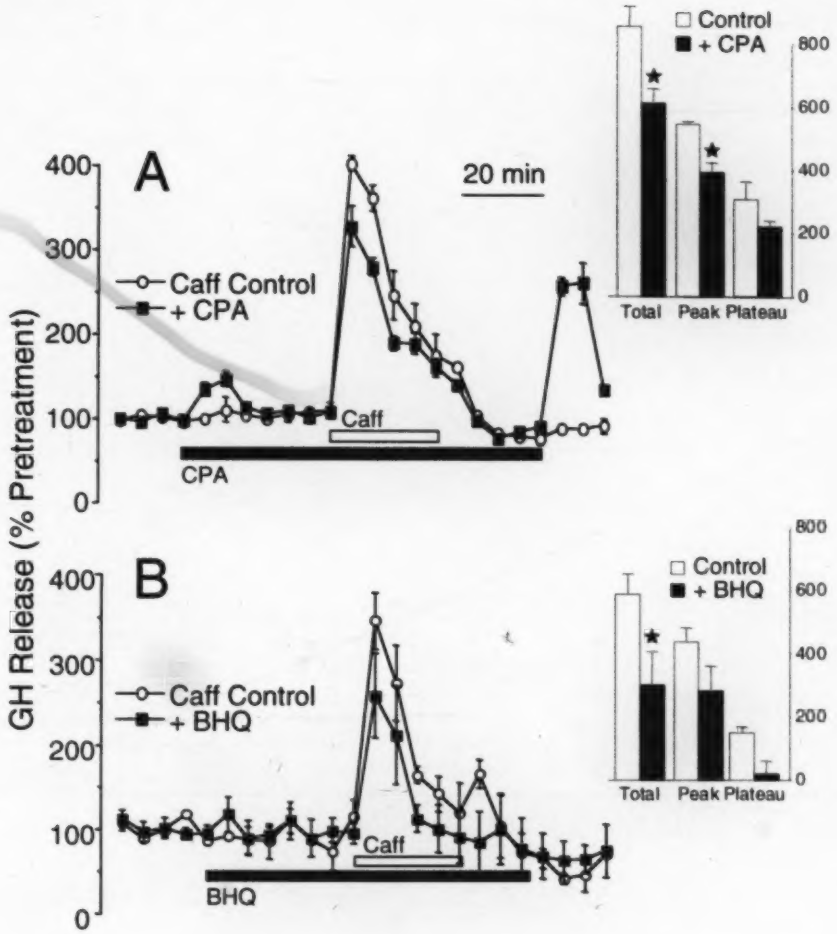

Figure 7.10. Effects of SERCA inhibitors on caffeine-stimulated GH release. GH release response to 10 mM caffeine (*light bars*) is only partially inhibited by 10 μM CPA (A) or 10 μM BHQ (B) applied as indicated *(dark bars). Insets* show quantified responses (CPA n=4; BHQ n=4).

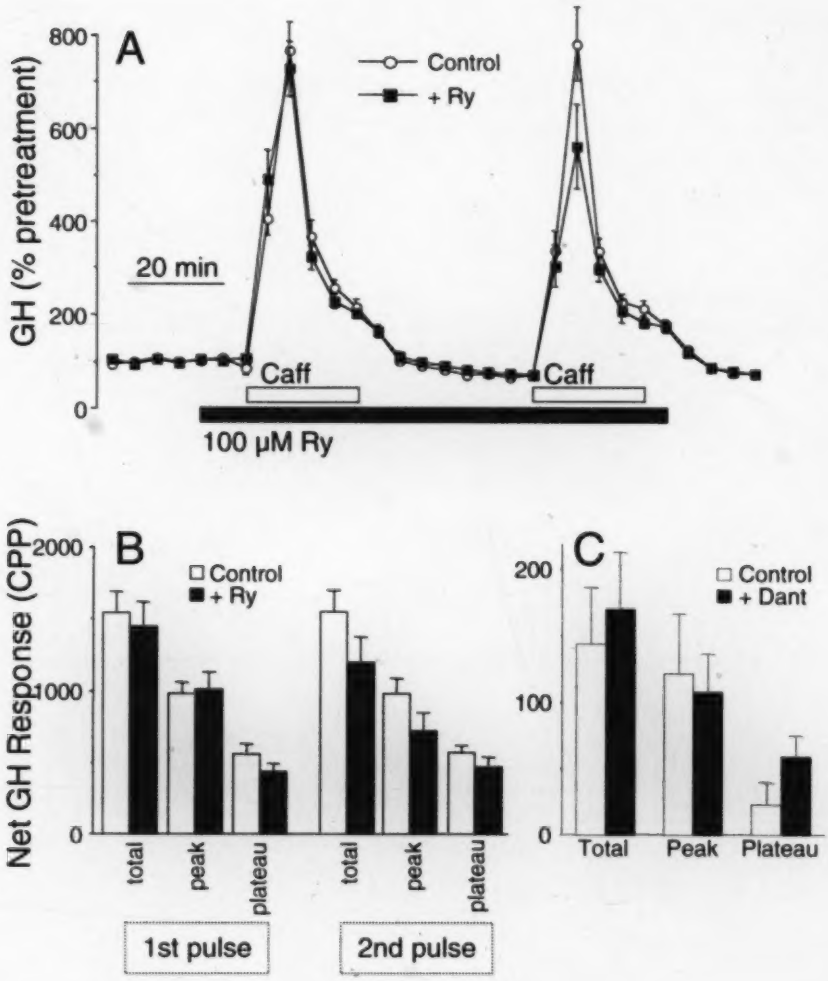

Figure 7.11. Caffeine-stimulated GH release does not require RyR. A) Treatment with 100 µM ryanodine (*black bars*) does not inhibit sequential GTH-II responses elicited by caffeine (*white bars*). B) Data from Part A are quantified (n = 8). C) Caffeine-stimulated GTH-II release is not inhibited by 50 µM dantrolene (30 minute pretreatment) in perifusion experiments similar in design to those shown in Figure 7.10. (n = 8).

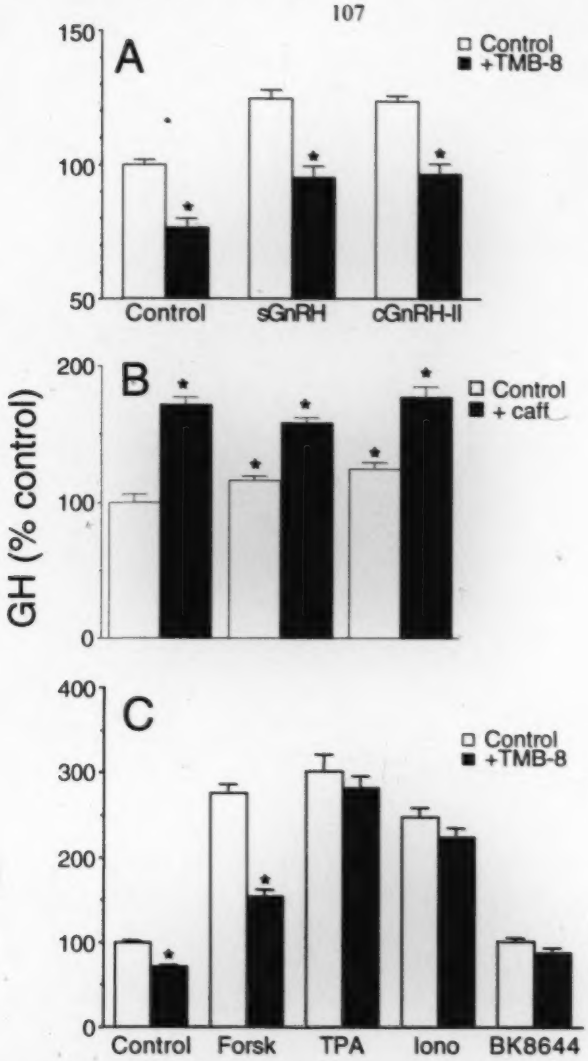

Figure 7.12. Involvement of intracellular Ca^{2+} stores in prolonged GH release. A) 2-hour incubation with 100 μM TMB-8 reduced basal GH release and GH release responses to 100 nM sGnRH or 100 nM cGnRH-II (n=16-24). B) 100 nM sGnRH or 100 nM cGnRH-II do not evoke additive GH release in the presence of 10 mM caffeine. C) TMB-8 reduced forskolin (10 μM)- stimulated GH release. In contrast, TMB-8 does not affect downstream components of the 1 μM- or 10 μM (not shown) ionomycin-stimulated GH release pathways. A lack of effect of 10 μM BAYK 8644 indicates that VGCC activity is not functionally significant during basal conditions (n=24-36). Significant difference from controls is indicated by a *star*.

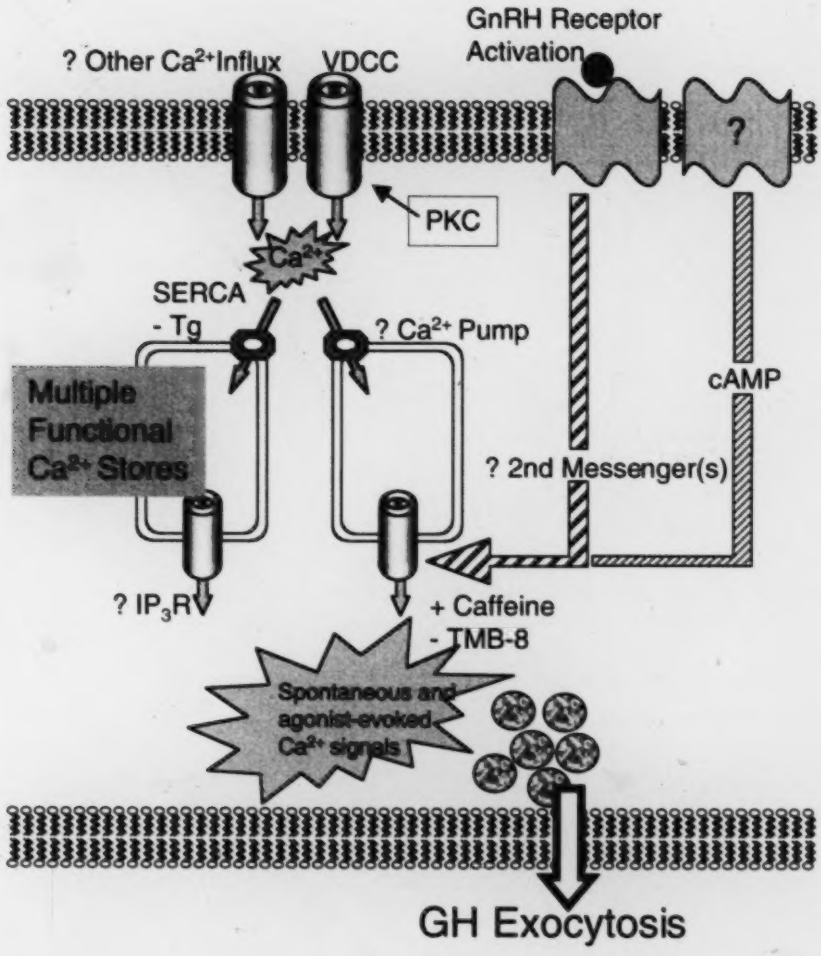

Figure 7.13. Working model of the role of intracellular Ca^{2+} stores in basal and GnRH-stimulated GH release in goldfish somatotropes. GnRH receptor occupation, and possibly agonists that use cAMP-dependent signalling pathways (perhaps dopamine or PACAP; Chang et al., 2000), lead to the activation of caffeine/TMB-8 sensitive intracellular Ca^{2+} release channels. These channels are found on Ca^{2+} stores that are highly-sensitive to manipulation of extracellular Ca^{2+} availability and may be rapidly depleting. These stores spontaneously release Ca^{2+} in some somatotropes, leading to increased basal GH release. The refilling mechanism is unknown, but known SERCA proteins (sensitive to Tg, BHQ or CPA) may not play a significant role. The involvement of protein kinase C is likely downstream or independent of the activation of TMB-8-sensitive Ca^{2+} stores. Stimulatory drugs are indicated by plus signs and inhibitory drugs are indicated by minus signs.

Chapter 8.

Evidence for plasticity of ryanodine-sensitive Ca^{2+} signalling systems

Several types of receptor/channels have been implicated in the release of Ca^{2+} from intracellular sources. Among these, the IP_3R and the RyR are the most extensively characterized (Pozzan et al., 1994; Ehrlich et al., 1994; Berridge, 1997). Both IP_3R and RyR are found in at least three different isoforms, each with distinct Ca^{2+} release kinetics (Chapter 1). Strategic expression of these molecules on the endoplasmic reticulum (ER), and possibly other organelles, may lead to physiologically important differences in the spatial and temporal characteristics of Ca^{2+} signals in a given cell (Petersen et al., 1994; Berridge, 1997; Johnson and Chang, 2000a; Chapter 1)

There is mounting evidence that RyRs (especially RyR2 and RyR3) play a critical role in several types of neuronal plasticity. These include, entrainment of the circadian clock of the superchiasmic nucleus, spatial learning, memory, LTP and LTD (Wang et al., 1996; Cavallaro et al., 1997; Ding et al., 1998; Balschun et al., 1999; Reyes-Harde et al., 1999; Zhao, et al., 2000). Can these novel mechanisms for plasticity be extended to include regulation of long-term changes in seasonal cycles that control reproduction and growth? The presence and activity of RyR in the pituitary is still incompletely understood and controversial for some cell types. Sundaresan and co-workers (1997) found RyR2 and RyR3 mRNA in the rat pituitary, and proposed its involvement in GTH synthesis and release based on experiments with ruthenium red. However, these results have not been confirmed using ryanodine, which is a more specific probe for the RyR. Furthermore, several other groups have not found evidence of Ca^{2+} stores sensitive to ryanodine or caffeine in single gonadotropes (Kukuljan et al., 1994; Hille et al., 1995). In rat somatotropes, evidence against a role for CICR involving ryanodine-sensitive Ca^{2+} stores (Tomic et al., 1999) is countered by reports of RyR involvement in the signal transduction of GHRH and GHRP-6 (Herrington and Hille, 1994; Petit et al., 1999). Perhaps RyR stores are present in pituitary cells, but do not perform the classical CICR role.

Multiple forms of GnRH occur in all classes of vertebrates, including humans (White et al., 1997). However, the signal transduction cascades of multiple GnRHs have been compared primarily in the goldfish model, where the signalling systems mediating the actions of sGnRH and cGnRH-II have been extensively studied. Although the *in vitro* and *in vivo* sensitivity of gonadotropes to GnRH stimulation varies with the reproductive status of the fish, both endogenous neuropeptides are important physiological regulators of GTH-II release throughout the year (Murthy et al., 1994; Chang et al., 1996). Likewise, the relative effectiveness of both sGnRH and cGnRH-II in the stimulation of GH release also fluctuates throughout the year, both *in vivo* and *in vitro* (reviewed in Chang et al., 1996; Peter and Chang, 1999). Results from Chapters 5, 6, and 7 in this thesis have demonstrated a dominant role for intracellular Ca^{2+} stores in the secretagogue signalling of these two endogenous forms of GnRH in both goldfish gonadotropes and somatotropes throughout the reproductive cycle (Johnson and

Chang, 2000b; Johnson et al., 2000; Chapters 5,7). Partial characterization of GnRH-sensitive Ca^{2+} signalling systems in both cell types reveals important differences compared to classical IP_3-mediated pathways, and considerable differences between gonadotropes and somatotropes in the goldfish.

The observation, in both cell types, that the GnRH-sensitive Ca^{2+} stores appeared to differ from the classical Tg/IP_3/agonist-sensitive Ca^{2+} pool, as well as the findings that caffeine evoked massive GTH-II and GH release (Johnson and Chang, 2000b; Johnson et al., 2000; Chapters 5,7), led us to investigate the role of ryanodine-sensitive Ca^{2+} stores in basal and GnRH-stimulated GTH-II and GH release. In this chapter, I report that RyR-expressing Ca^{2+} stores are differentially coupled to GnRH signalling in goldfish pituitary cells, depending on the reproductive stage and on the GnRH isoform. In addition, novel evidence is provided that these ryanodine-sensitive Ca^{2+} stores differ from classical caffeine-sensitive Ca^{2+}-induced Ca^{2+} release stores.

Results

The most reliable pharmacological probe for the RyR is ryanodine. Ryanodine binds to the RyR (in the open conformation) and locks it in a sub-conductance state, characterized by low conductance and high open probability (Rousseau et al., 1987; Ehrlich et al., 1994). A hallmark characteristic of ryanodine action is that it stimulates the receptor at nanomolar concentrations but inhibits ryanodine-sensitive Ca^{2+} stores at micromolar doses (Ehrlich et al., 1994; Hong et al., 2000). We took advantage of this property and applied a wide range of ryanodine concentrations to mixed cultures of goldfish pituitary cells. Indeed, ryanodine-sensitive Ca^{2+} stores were found to be coupled to both GTH-II and GH release in a dose-response profile consistent with the known mechanisms of ryanodine (Fig. 8.1). Significant stimulation of hormone release was not observed at 'blocking' doses of ryanodine (1-10 μM) over the 2-hour period. On the other hand, the magnitude of the stimulatory effect of low doses of ryanodine is similar to the GTH-II- and GH-releasing activity of a maximal dose (100 nM) of GnRH in static culture (typically ~150% of control; e.g. Figs. 5.1, 7.1), indicating that these responses are of a physiologically-relevant magnitude. In agreement with the proposed activity of low doses of ryanodine and the hormone release data, application of 0.1 nM ryanodine caused small, but consistent, increases in $[Ca^{2+}]_c$ in morphologically-identified gonadotropes and somatotropes loaded with Fura-2 (n=5, data not shown).

Having shown that ryanodine-sensitive Ca^{2+} stores can regulate hormone secretion in both cell types, we asked whether RyR are involved in GnRH-stimulated GTH-II or GH release. The results of these studies fell naturally into groups based on the reproductive status of the donor fish. In gonadotropes, stage-related differences were obvious in the GnRH-stimulated and basal hormone release profiles of the control columns. First, quantified GTH-II responses to 5-minute GnRH challenge were approximately 3 times greater in cells from mature fish, when compared with sexually regressed fish (Fig. 8.2). Second, there were certain hallmark differences in the characteristics of basal hormone release. For example, there was a more pronounced decline in basal GTH-II release over the

course of the experiments using cell from sexually regressed animals. These characteristics have been consistently observed over many years in a variety of similar experiments in our laboratory (e.g. Lo and Chang, 1998; JP Chang and JD Johnson unpublished observations). In spite of these known seasonal differences in GnRH-evoked and basal hormone release, dramatic seasonal changes in the signal transduction underlying these processes have not been reported, until now. Figure 8.2 shows that the effect of inhibitory doses of ryanodine (10 μM) on GnRH-stimulated GTH-II release was also strongly seasonal. In the presence of ryanodine, GTH-II responses to either sGnRH or cGnRH-II in cells from sexually regressed fish were abolished (Fig. 8.2 A,C). However, in experiments with cells from sexually mature fish, ryanodine only blocked cGnRH-II-stimulated GTH-II release, while GTH-II responses to sGnRH were unaffected. Taken together, these data indicate that GnRH-stimulated GTH-II release is mediated by RyR, except in the sexually mature stage, where sGnRH signalling apparently switches to different intracellular Ca^{2+} store(s).

Goldfish somatotropes also display unique seasonal behavior, *in vitro*, including a dramatically increased responsiveness to GnRH in cells prepared from sexually mature fish. Interestingly, the involvement of RyR in GnRH-stimulated GH release, measured from the same experiments from which GTH-II was measured (see above), was entirely different. Ryanodine had no effect on GH release stimulated by either GnRH during the period of sexual maturity (Fig. 8.2). In experiments on cells from sexually regressed fish, sGnRH-evoked GH release was also independent of ryanodine-sensitive Ca^{2+} stores; in contrast, ryanodine blocked cGnRH-II-stimulated GH release. These novel findings were unexpected given that signalling differences between sGnRH and cGnRH-II had not previously been reported in goldfish somatotropes. These results demonstrate that GnRH signalling in somatotropes does not involve the release of Ca^{2+} from ryanodine-sensitive intracellular stores, except in the case of cGnRH-II-stimulated GH release from cells isolated from sexually regressed fish.

Since acute GH release evoked by both GnRHs is totally and equally dependent on intracellular Ca^{2+} stores (Johnson and Chang, 2000b; Chapter 7), the differential sensitivity of sGnRH- and cGnRH-II-stimulated GH release to ryanodine in regressed fish suggested the involvement of other pharmacologically distinct Ca^{2+} stores, at least at this stage of gonadal maturation. Since IP_3 receptor-linked Ca^{2+} pools are thought to mediate intracellular Ca^{2+} release from agonist-sensitive stores in most cell types (Pozzan et al., 1994), the possible involvement of IP_3-dependent mechanisms in sGnRH and cGnRH-II action on GH release was examined. Indeed, sGnRH-stimulated GH release (from cells isolated from regressed fish) was significantly reduced in the presence of 1 μM xestospongin C (Fig. 8.3), a potent and selective inhibitor of IP_3 receptors (Gafni et al., 1997). Conversely, cGnRH-II-evoked GH secretion was unaffected. Taken together, results from the ryanodine and xestospongin C experiments provide strong evidence that Ca^{2+} signalling leading to GH exocytosis is mediated by agonist-specific intracellular Ca^{2+} stores in cells from sexually regressed goldfish. The exact identity of the GnRH-sensitive Ca^{2+} store(s) in somatotropes from sexually mature fish, remains to be elucidated.

Next, we began to deduce the pharmacological properties of ryanodine signalling leading to GTH-II and GH exocytosis. In the CNS, some responses attributed to RyR can be blocked by dantrolene, an inhibitor of the skeletal/RyR1 isoform (Simpson et al., 1995). Dantrolene (50 μM) did not affect GTH-II or GH release evoked by either of the GnRHs (Fig. 8.4). Similarly, caffeine evoked GTH-II or GH responses are not inhibited by dantrolene (Chapters 6,7). These results suggest that RyR1 is not involved in GnRH-stimulated hormone release in the goldfish pituitary and, by elimination, implicates RyR2 (heart) or RyR3 (brain) isoforms.

Do the ryanodine-sensitive components of GnRH signalling reflect a Ca^{2+}-induced Ca^{2+} release phenomenon or do these Ca^{2+} stores occupy a role similar to classical IP_3-sensitive Ca^{2+} stores by acting as the initial trigger for agonist Ca^{2+} signalling? To test these possibilities, the ability of ryanodine to block hormone release elicited by direct modulation of second messenger cascades was examined. Both cAMP and PKC have been implicated as elements upstream of extracellular Ca^{2+} influx in the signal transduction of multiple neuroendocrine regulators of hormone release in goldfish gonadotropes and somatotropes (Chang et al., 1996). Although, PKC is an integral component of GnRH signalling in both cell types, GTH-II and GH release evoked by 10 nM TPA was unaffected by 10 μM ryanodine (Fig. 8.5), suggesting that ryanodine-sensitive Ca^{2+} stores are not downstream of PKC activation. On the other hand, the cAMP/PKA pathway has been proposed to mediate the secretagogue actions of dopamine and PACAP on GH release (Wong et al., 1994; Wong et al., 1998), as well as the effects of PACAP on GTH-II release (JP Chang, NR Wirachowsky, P Kwong, JD Johnson, unpublished results), by increasing the current through VGCCs (reviewed in Chang et al., 2000). Treatment with 10 μM ryanodine did not affect 10 μM forskolin-induced GTH-II secretion, and had only a minor effect on GH release. Likewise, inhibition of ryanodine receptors had no effect on hormone release evoked by 1 mM 8-bromo-cAMP (Fig. 8.5). Ryanodine also did not block GH release evoked by a dopamine D1 agonist. 10 μM SKF38393-stimulated GH release (239.5 ± 20.4 % of basal) was not significantly affected in the presence of ryanodine (226.7 ± 16.1 % of basal; n=16). Most importantly, ryanodine did not block either GTH-II or GH release when $[Ca^{2+}]_c$ is elevated directly, since the responses to 10 μM ionomycin or 10 μM BayK 8644 were not affected by ryanodine treatment (Fig. 8.5). These data clearly argue against a prominent CICR role for ryanodine-sensitive intracellular Ca^{2+} stores in these cell types. Collectively, these results strongly suggest that Ca^{2+} signalling through ryanodine receptors occurs early after the activation of GnRH receptors, a role hitherto generally ascribed to IP_3 receptor-linked Ca^{2+} pools.

How are ryanodine-sensitive stores related to those activated by caffeine? If caffeine and ryanodine share the same target in gonadotropes and somatotropes, then their actions would be expected to be similar. However, several clues suggested that this may not be the case. In gonadotropes, the caffeine-sensitive Ca^{2+} pool was only involved in sGnRH-stimulated GTH-II secretion (Johnson et al., 2000; Chapter 5), while ryanodine-sensitive Ca^{2+} stores are involved in the signalling of both GnRHs (this Chapter). In somatotropes, caffeine abolishes both

sGnRH- and cGnRH-II-evoked GH release (Johnson and Chang, 2000b; Chapter 7), under conditions when ryanodine is without effect (this Chapter). These contrasting findings led us to investigate whether hormone release evoked by caffeine, which activates RyR in many systems (Ehrlich et al., 1994), was sensitive to ryanodine in goldfish gonadotropes and somatotropes. As in previous chapters, 10 mM caffeine alone evoked massive hormone release from both cell types (Fig. 8.6). 10 μM ryanodine had no effect on caffeine-stimulated GTH-II release and caused only a minor reduction of caffeine-evoked GH release (Fig. 8.6). These results indicate that the intracellular Ca^{2+} stores modulated by caffeine and ryanodine are largely independent, from a functional standpoint, in these two cell types.

Finally, we asked whether caffeine-sensitive intracellular Ca^{2+} stores were more highly coupled to GTH-II or GH secretion during specific periods of the seasonal reproductive cycles of goldfish. Caffeine-stimulated hormone release responses were quantified from experiments compiled over a 2.5 year period. As was the case with the coupling of ryanodine-sensitive Ca^{2+} stores to agonist-evoked hormone release, clear seasonal differences in the relative effectiveness of caffeine are evident in both GTH-II and GH release responses (Fig. 8.7). The *in vitro* basal GTH-II and GH secretion also varied with the seasonal reproductive status of the donor fish. Whereas basal release of GTH-II is greatest from primary cultures of pituitary cells prepared from fish in late stages of gonadal recrudescence, basal GH release *in vitro* is highest in the fall when the fish are sexually regressed (Fig. 8.7). The maximum response to caffeine is found in the same period as the peak basal GTH-II release in gonadotropes. Interestingly, the peak effectiveness of caffeine may precede the elevation in basal GH release. Together with the other data presented in this report, these results suggest that the physiological function of multiple intracellular Ca^{2+} stores may be linked to the seasonal reproductive state, and that these effects persist in primary cultures of pituitary cells.

Discussion

Differential roles of RyR in GnRH signalling: dependence on cell type, GnRH isoform and reproductive status

The findings of the present study, in which the stimulatory actions of low doses of ryanodine were exploited (e.g. Hong et al., 2000), have demonstrated that RyR-expressing intracellular Ca^{2+} stores can be coupled to hormone release in dispersed goldfish pituitary cells. Furthermore, the present results show that GnRH-stimulated hormone secretion is mediated by ryanodine-sensitive intracellular Ca^{2+} stores under specific conditions. To my knowledge, this is the first report suggesting a role for RyR in pituitary hormone release in any non-mammalian vertebrate. Most importantly, this is a novel description of functional plasticity of pituitary agonist-sensitive Ca^{2+} signalling systems. The involvement of RyR in GnRH signalling is conditional on the reproductive status of the goldfish, and is specific with respect to GnRH isoform under certain conditions.

The seasonality of RyR involvement in the present study is particularly robust (either 'on' or 'off'). While ryanodine-sensitive mechanisms are necessary

for GTH-II responses to both GnRHs in experiments with cells from regressed fish, sGnRH completely looses this sensitivity in cells from sexually mature fish. RyR mediates cGnRH-II, but not sGnRH, action on GH release, but only in cells from sexually regressed fish. These data clearly illustrate plasticity in the recruitment of ryanodine-sensitive Ca^{2+} pools into the signalling systems controlling regulated exocytosis in both cell types. Since acute GnRH-stimulated hormone release, from both gonadotropes and somatotropes, is entirely dependent on intracellular Ca^{2+} stores (Johnson and Chang, 2000b; Johnson et al., 2000; Chapters 5,7), the RyR-independent periods in both cell types likely represent the recruitment of additional intracellular Ca^{2+} pools. Indeed, studies on other cell types (reviewed in Chapter 1), as well as the data described in this thesis suggest that cells may possess multiple agonist-sensitive Ca^{2+} stores. The involvement of these novel Ca^{2+} stores in several aspects of the secretory pathway, makes them important candidates for seasonal changes in neuroendocrine regulation. Further work may also be directed towards understanding which Ca^{2+} stores mediate GnRH-stimulated hormone release during the transition between reproductive stages.

Switching between multiple intracellular Ca^{2+} stores, each with particular kinetics of Ca^{2+} release and functional coupling to exocytosis, represents a novel form of cellular plasticity. Long-term cellular plasticity may also result from changes in ER morphology. Consistent with such a possibility, Subramanian and Meyer (1997) have shown that the restructuring of the ER can be quite rapidly and is regulated by Ca^{2+}. Using electron microscopy to study the ultrastructure of goldfish gonadotropes and somatotropes, several investigators have noted that modification of ER morphology and increases in ER volume are prominently associated with the onset of sexual maturity. Changes in both parameters are also seen with steroid-treatment or ovariectomy (Nagahama, 1973; Kaul and Vollrath, 1974). Recent *in vitro* experiments have shown that gonadal steroids can potentiate GnRH-stimulated GTH-II release (Lo and Chang, 1998) at the level of the pituitary, through the enhancement of post-receptor signalling. Although the exact mechanism of this effect is unknown, the possibility that GnRH-signalling becomes more highly coupled to GTH-II exocytosis through steroid-induced modulation of ryanodine-sensitive Ca^{2+} pools is an interesting and testable hypothesis. Future work is required to clarify the role of RyR in the reproductive cycle of goldfish. Conducting separate experiments on male and female goldfish may also lead to important insights.

One curious result of the present study is the finding that high (inhibitory) doses of ryanodine evoked marked changes in basal hormone release under some conditions. These effects were seen in cells from sexually matured fish which were pre-exposed to GnRH, but not in naive cells in experiments performed during the same reproductive stage (compare Fig. 8.2 D, F, H with Fig. 8.6). The mechanisms of the increases in hormone release seen in GnRH-pretreated cells are not yet known. One might speculate that they arise from the coupling of exocytosis to Ca^{2+} signals generated as a result of homeostatic responses when Ca^{2+} fluxes through the RyR are disrupted. Consistent with such a hypothesis, we have recently shown that compensatory increases in Ca^{2+} and GTH-II release

occur when Ca^{2+} entry into the cytosol from VGCC or TMB-8-sensitive Ca^{2+} stores are limited (Johnson et al., 2000; see Chapter 5). In any case, these results further illustrate that the recruitment of ryanodine-sensitive Ca^{2+} stores, as well as the consequence of their activation, can be modulated by neuroendocrine modulators. This and the observed seasonality of RyR involvement in GnRH signal transduction are consistent with the proposed role of RyR in other forms of cellular plasticity.

As described in the introduction to this Chapter, several recent studies have implicated RyR (especially RyR2, RyR3) in several forms of neuronal plasticity (Cavallaro et al., 1997; Ding et al., 1998; Balschun et al., 1999; Zhao, et al., 2000; Balschun et al., 1999; Reyes-Harde et al., 1999). The exact link between RyR and cellular plasticity is not known; one might speculate that differential recruitment of RyR-expressing Ca^{2+} stores at pre-synaptic sites may modulate signal transduction coupling to exocytosis. Alternatively, changes in RyR involvement at post-synaptic sites may control cell responsiveness. In this regard, it is worth noting that goldfish gonadotropes and somatotropes, unlike the analogous cell types in mammals, are *bona fide* post-synaptic cells, receiving direct hypothalamic innervation (reviewed in Ball, 1981; Chang et al., 1996; Peter and Chang, 1999). Although data presented herein do not address the exact mechanisms by which RyR modulate cellular plasticity, they offer clues as to the mechanisms and potential complexity of some types of long- and short-term changes in cellular responsiveness to external stimuli. To our knowledge, a clear and dramatic switch in the coupling of intracellular Ca^{2+} signalling systems to a physiological output (i.e. hormone secretion) has not been demonstrated previously.

Relationship of RyR with known signalling pathways and CICR

Many of the studies, including those conducted on rat gonadotropes, have looked for RyR in the context of its possible involvement in CICR. The relationship between RyR and CICR was investigated using activators of post-receptor signalling components known to mediate agonist-evoked hormone release in goldfish gonadotropes and somatotropes. Activation of PKC and cAMP/PKA pathways have been proposed to modulate VGCC-mediated Ca^{2+} entry, thereby elevating $[Ca^{2+}]_c$ (reviewed in Chang et al., 1996). Such increases in $[Ca^{2+}]_c$ may lead to CICR, a function generally attributed to RyR (Pozzan et al., 1994; Petersen et al., 1994; Berridge, 1997). Results from the present study demonstrate that ryanodine-sensitive Ca^{2+} stores are not downstream of PKC-dependent events. Since PKC is an essential part of GnRH signalling in both cell types (reviewed in Chang et al., 1996), RyR activation may represent an early event (upstream of PKC) in GnRH signal transduction. It appears that dopamine (D1)/cAMP-stimulated hormone release is also largely independent of RyR. The possible existence of a small downstream RyR component in the cAMP pathway in somatotropes (the small inhibition of forskolin-stimulated hormone release) is not supported by the observation that 8-bromo-cAMP-stimulated GH release is unaffected. Instead, the result with forskolin may suggest the minor presence of a Ca^{2+}-sensitive adenylate cyclase. Further work will be required to determine

whether RyR is involved in the signalling of other neuroendocrine regulators of GTH-II and GH release. We hypothesize that in goldfish gonadotropes and somatotropes, ryanodine-sensitive Ca^{2+} stores (when involved) are unlikely to participate in CICR, but are involved in the initiation of GnRH Ca^{2+} signalling.

In any case, the ryanodine-sensitive intracellular Ca^{2+} stores of goldfish gonadotropes and somatotropes clearly do not correspond to classical caffeine-sensitive stores known to mediate CICR in many cells types. Although the activity of ryanodine and caffeine overlap in the majority of cases, there are some notable exceptions in the literature (e.g. Langley and Pearce, 1994). One example of a caffeine-insensitive RyR is the TGF-β-sensitive RyR of mink, which is thought be a RyR3 (Giannini et al., 1992; Meissner, 1994). On the other hand, Conkin and co-workers (2000) found that the Ca^{2+} release from RyR3, but not RyR1, was enhanced by caffeine treatment in embryonic skeletal muscle. Further work will be required in order to characterize the dantrolene- and caffeine-insensitive RyR isoform involved in GnRH-stimulated hormone release from the goldfish pituitary.

The large degree of independence of caffeine and ryanodine action was unexpected, and means that caffeine cannot be used as a reliable probe for ryanodine-sensitive Ca^{2+} stores in goldfish gonadotropes and somatotropes. Nonetheless, several interesting inferences can be made from the caffeine data, where the very large data set allows experiments to be grouped by month. The basal hormone release values show the expected *in vitro* seasonal distribution. In sexually mature or pre-spawning fish, the high basal release of GTH-II from cultured cells mirrors the *in vivo* serum levels, which are also highest in the spring. The high *in vitro* basal GTH-II secretion in the spring probably reflects the combination of increased positive feedback from gonadal steroids and the loss of tonic inhibitory dopamine influence, which is also highest in the spring (Habibi et al., 1989; Lo and Chang, 1998). The coupling of caffeine-sensitive intracellular Ca^{2+} stores to hormone release also appears to be related to reproductive status, with the largest caffeine-stimulated GTH-II release found in cells from sexually mature fish. Previously, we have shown that the stimulation-secretion coupling of sGnRH, but not cGnRH-II (which does not use caffeine-sensitive Ca^{2+} stores), is enhanced at this reproductive stage (Lo and Chang, 1998). This correlation suggests that sGnRH may recruit caffeine-sensitive Ca^{2+} stores, which seem to be very highly coupled to GTH-II exocytosis (especially during sexual maturation). A hypothetical scheme, summarizing the current understanding of seasonal changes in the efficacy of sGnRH, cGnRH-II and caffeine stimulation of GTH-II release in goldfish gonadotropes is displayed as Figure 8.8. The involvement of RyR (and IP_3) during various times of the year (where studied) is also illustrated, for comparison.

The seasonal pattern of basal GH release is reversed when compared to GTH-II. In the case of somatotropes, basal hormone release does not parallel serum GH levels over the year (serum GH is highest in sexually mature fish; Peter and Chang, 1999). The high basal GH secretion seen in the fall, *in vitro*, likely reflects the withdraw from tonic somatostatin inhibition, which has been proposed to be maximal at this time of the year (Peter and Chang, 1999). Caffeine-

stimulated GH release displays a biphasic seasonal profile. Caffeine-sensitive Ca^{2+} stores are most highly coupled to GH exocytosis in September, which is notable because it is the period of peak sensitivity to the stimulatory actions of dopamine through D1 receptors (Peter and Chang, 1999). Although we have ruled out the involvement of RyR in D1-stimulated GH release in the present study, we have no evidence against the involvement of caffeine-sensitive Ca^{2+} stores in D1 action. Recent experiments have identified some similarities, as well as some differences, between caffeine and D1 signalling in somatotropes (CJH Wong, JD Johnson, WK Yunker and JP Chang, unpublished). Unlike the case in gonadotropes, caffeine-sensitive Ca^{2+} stores occupy a major role in the signalling of both GnRHs in goldfish somatotropes. The maximum GnRH effectiveness (observed in sexually mature fish) correlates with period where the second largest caffeine-stimulated GH responses are found. Taken together, these results suggest that caffeine-sensitive Ca^{2+} stores, and their effective coupling to exocytosis, may be important targets of neuroendocrine regulation and provide a starting point for future investigations. The efficacy and seasonality of caffeine make it a useful tool for the investigation of physiological involvement of intracellular Ca^{2+} stores, but not those sensitive to ryanodine, in these two cell types.

If RyR Ca^{2+} stores are not activated by CICR, it is not clear how GnRH receptor occupation is coupled to ryanodine-sensitive Ca^{2+} stores in goldfish pituitary cells. Both cADPr and nitric oxide have been implicated in RyR-dependent signalling cascades in a number of systems (e.g. Reyes-Harde et al., 1999; Willmott et al., 2000). Preliminary experiments using sGnRH indicate that evoked GTH-II release is not blocked by the membrane-permeant cADPr analog, 8-bromo cADPr, whereas the GH response is inhibited (JD Johnson and JP Chang, unpublished). We have also shown that nitric oxide is an important mediator of GnRH signalling in goldfish somatotropes (Uretsky and Chang, 2000) and gonadotropes (AD Uretsky and JP Chang, unpublished data). A NO-cGMP-RyR pathway is known to play a role in ryanodine-sensitive synaptic plasticity (Reyes-Harde et al., 1999). Experiments designed to evaluate the role of this pathway in goldfish pituitary hormone release are required.

Ryanodine-sensitive Ca^{2+} stores as one of several pharmacologically distinct stores

In addition to implicating RyR in the initiation of GnRH-evoked Ca^{2+} signals, the present study expands our understanding of the multiplicity of pharmacologically and functionally distinct non-mitochondrial Ca^{2+} stores in endocrine cells. This is also the first report of agonist-specific Ca^{2+} stores in goldfish somatotropes. Specifically, Ry-sensitive Ca^{2+} stores are only coupled to cGnRH-II signalling during the sexually regressed stage, although they are likely present throughout the reproductive cycle, since activation of RyR by low doses of ryanodine can couple to GH release during all times of the year. In the case of sGnRH-stimulated GH release from cells in a sexually regressed state, the present study demonstrates that at least one of the 'other' Ca^{2+} pools is linked to IP_3R. Although IP_3-mediated release is common to many agonist-signalling pathways, these stores display a unique pharmacology in goldfish somatotropes, since

GnRH-stimulated GH release is not sensitive to SERCA inhibitors (Johnson and Chang, 2000b; Chapter 7). Further experimentation will be required to elucidate the involvement of this store in GnRH signalling from somatotropes of sexually mature fish. Nonetheless, this is the first demonstration of differential signal transduction between the GnRHs in somatotropes. The participation of multiple pharmacological classes of intracellular Ca^{2+} stores in the physiological signalling of single cell type is not without precedent (see discussion of CCK signalling in pancreatic acinar cells; Chapters 1,5; Cancela et al., 1999). Although signal coding through specific activation of multiple Ca^{2+} stores can generate the complexity required for the independent regulation of cellular functions by Ca^{2+}, examples of such signalling are few. This is the first study to suggest that such complexity may be important in pituitary cells.

Comparison of RyR involvement in fish and mammalian pituitary cells

Whether conclusions from the present study can be generalized to mammalian pituitary cell systems, will require further investigation. On one hand, there are some indications that GnRH signalling has species-specific features. In rat gonadotropes, application of mGnRH leads to high amplitude Ca^{2+} signals, which often involve dramatic oscillations, mediated by IP_3 receptors (Hille et al., 1995). In contrast, GnRH-evoked Ca^{2+} signals in goldfish gonadotropes and somatotropes are very different, especially with respect to their temporal profile. GnRH-induced Ca^{2+} signals in goldfish have a much lower global amplitude and do not display the same kind of high amplitude oscillations (Johnson et al., 1999; Chapter 4) found in rat pituitary cells. Instead, these Ca^{2+} signals are more characteristic of Ca^{2+} release from intracellular stores in non-excitable cell types. Indeed, acute GnRH signalling is independent of plasma membrane excitability in goldfish (Johnson and Chang, 2000b; Johnson et al., 2000; Chapters 5,7). If the difference in excitation-secretion coupling between goldfish and rat gonadotropes is the relative involvement of RyR and IP_3R, the differences between the kinetic properties (e.g. conductance) of these two channels may explain the differences in observed Ca^{2+} signals. Whereas high amplitude (>1 μM) oscillatory Ca^{2+} signals are commonly mediated by IP_3R, such events are not normally attributed to Ca^{2+} signalling systems involving RyR. Instead, in several cell types, activation of RyR leads to sub-micromolar Ca^{2+} signals which are not oscillatory in nature (e.g. Herrington and Hille, 1994; Paltauf-Doburzynska et al., 1998; Krizaj et al., 1999; Willmott et al., 2000).

To be sure, the role of RyR in rat gonadotropes is controversial. Most authors attribute agonist-evoked intracellular Ca^{2+} signalling in rat gonadotropes to a single IP_3 -sensitive pool filled by SERCA pumps (Kukuljan et al., 1994; Hille et al., 1995). Although RyR has been implicated in GTH secretion by Sundaresan and colleagues (1996), several groups found no evidence for RyR in signalling components related to GnRH action. For example, ryanodine (5 μM or 100 μM) did not modulate Ca^{2+}-activated K^+ channels (Kukuljan et al., 1994) in gonadotropes obtained from ovariectomized rats. Hille et al. (1995) has also reported non-involvement of RyR in gonadotropes from male rats. As in the present study, the reproductive state of the donor animals may be important.

Indeed, levels of RyR3, but not RyR2, mRNA varied with the reproductive cycle and were modulated by sex steroids in female rats (Sundersan et al., 1997). In addition, a recent report suggests that functional ryanodine-sensitive Ca^{2+} stores may be developmentally regulated (Zemkova and Vanecek, 2000). It is also notable that, in primary cultures of cortical neurons, RyR-mediated CICR disappears after a few days in culture (Mhyre et al., 2000). As was the case in our study, the group that implicated RyR in rat GTH release used freshly isolated cells, while the other groups maintain their dispersed cells for up to a week in culture (e.g. Hille et al., 1995). In light of our results, and upon close examination of the differences in experimental model systems, major discrepancies within the rat gonadotrope literature may be reconciled. Nevertheless, it is notable that $RyR3^{-/-}$ mice are reproductively viable (Takeshima et al., 1996), suggesting that RyR3 is non-essential, or that its role is assumed by another isoform or a different Ca^{2+}-release channel.

Summary and significance

Results from the present study clearly demonstrate the involvement of RyR as one of the mechanisms mediating GnRH signalling in goldfish gonadotropes and somatotropes. Furthermore, these data suggest that Ca^{2+} signalling systems can be more labile than previously appreciated in endocrine cells. The combination of the growing knowledge of the multiplicity of Ca^{2+} signalling pathways and the extended reproductive cycles of goldfish, make them an excellent model for the study of how changes in Ca^{2+} signalling at the molecular level may lead to alterations in sensitivity of the pituitary to neuroendocrine regulation, and eventually the physiology of the whole-animal.

At this time, we do not know if cyclic changes in the involvement of RyR in goldfish pituitary cells is due to, 1) increased expression of RyR, as is the case in long-term memory, or 2) enhanced physical or biochemical coupling of these Ca^{2+} stores to agonist-signalling pathways or to the exocytosis machinery. Since RyR has a large single channel conductance (Meissner, 1994), relatively few molecules would be needed to change the Ca^{2+} signalling characteristics of a cell. Once recruited, ryanodine-sensitive intracellular cellular stores may represent a very stable change in the stimulus-secretion coupling of cells. I propose that switching between states of functional RyR signalling may be a general mechanism for controlling intrinsic cyclic activity of cells, including those of the goldfish pituitary, over a wide range of time scales.

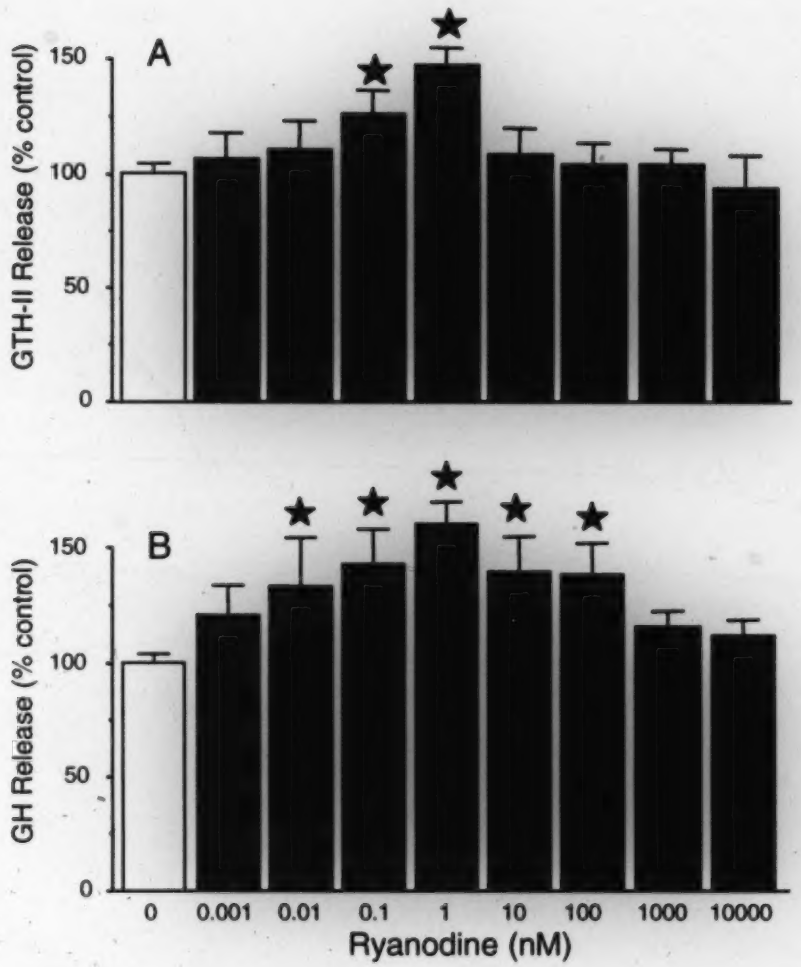

Figure 8.1. Low doses of ryanodine stimulated hormone release from goldfish pituitary cells *in vitro*. Dose-response profile of GTH-II (A) and GH (B) release from mixed populations of dispersed pituitary cells incubated for 2 hours with ryanodine at the dose indicated. Data from cell preparations from both sexually regressed and mature fish were similar and are therefore pooled (n=24). *Stars* indicate significant difference from control.

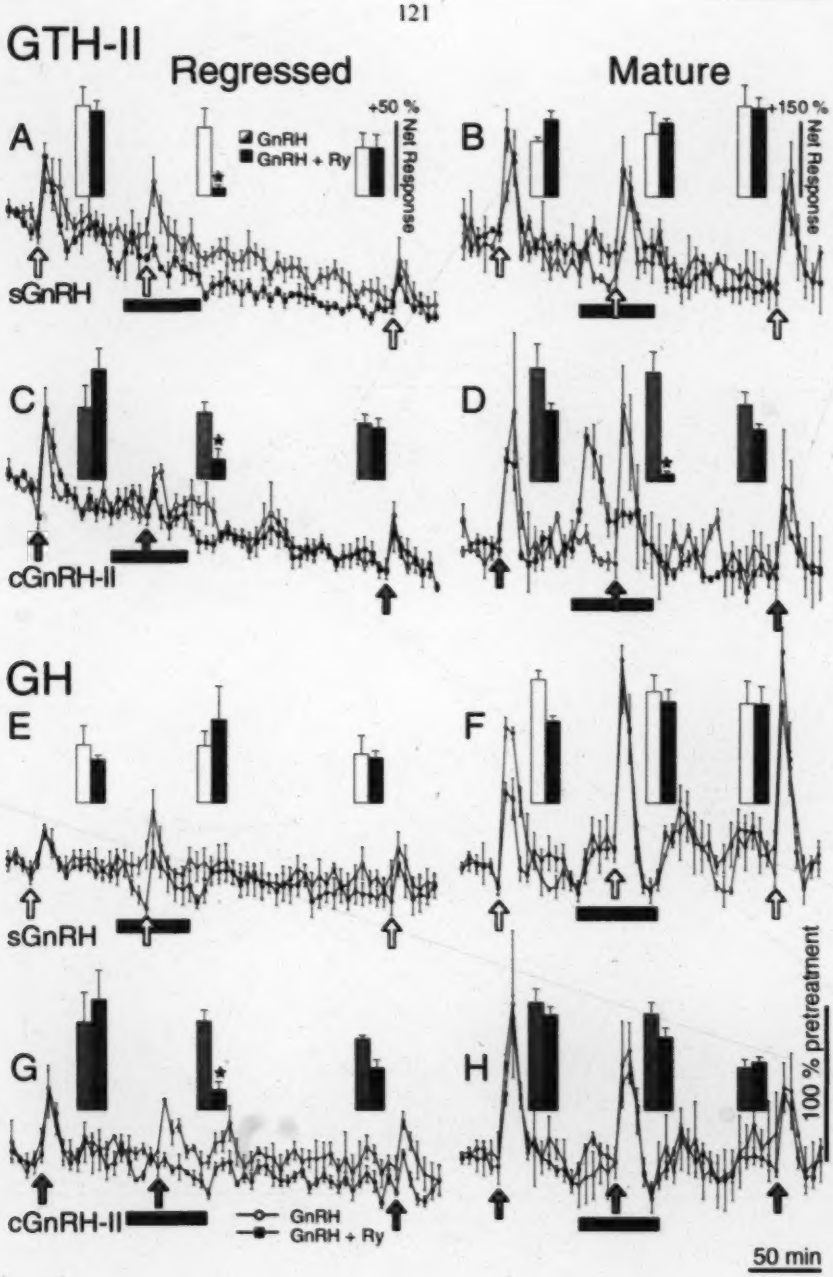
GTH-II
Regressed
Mature
A
B
C
D
GnRH
GnRH + Ry
+50 %
+150 %
Net Response
sGnRH
cGnRH-II
GH
E
F
G
H
100 % pretreatment
50 min

Figure 8.2. Conditional involvement of RyR in hormone release from goldfish pituitary cells. GTH-II (A-D) or GH (E-H) release in response to sequential applications of 100 nM GnRH in the presence (*filled circles*) or absence (*open circles*) of 10 μM ryanodine (*horizontal black bar*). 5-minute pulses of sGnRH (*light arrows*) or cGnRH-II (*dark arrows*) were added to cell perifusion columns as indicated (n=4 for each graph). Time scale bars in the lower right hand corner apply to all the perifusion profiles shown. Vertical bar graphs represent the hormone release response to GnRH, quantified as described in Materials and Methods (units = cumulative % pretreatment). Note that the scale bars for quantified hormone responses (indicated along the side of the upper panels) are different between experiments with cells from sexually regressed (left panels) and sexually mature fish (right panels). The units on these scale bars (and subsequent quantified hormone release responses in other figures) is cumulative % above baseline. *Stars* indicate significant difference from both parallel and sequential (within column) controls. In cases indicated by stars, hormone release responses to GnRH in the presence of ryanodine are also not significantly different from spontaneous fluctuations in hormone release during the same period in columns treated with ryanodine alone (i.e. missing the middle pulse of GnRH). For clarity, these columns were not shown.

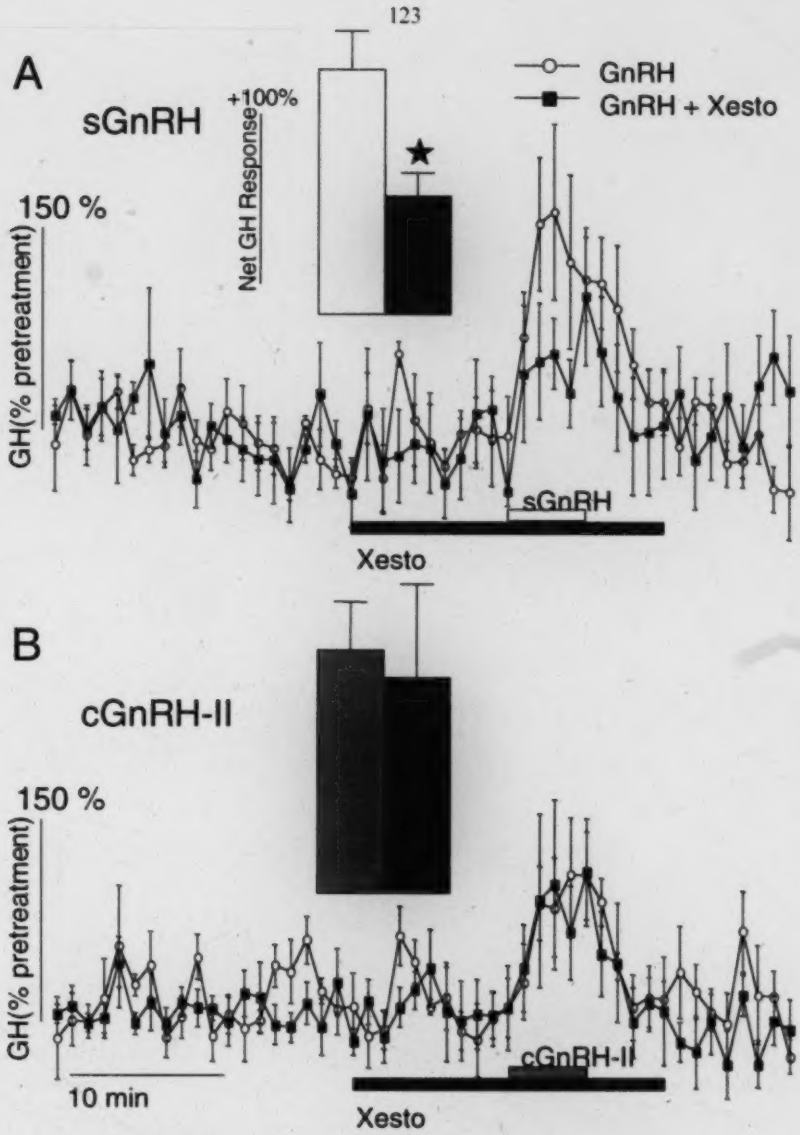

Figure 8.3. sGnRH-stimulated GH release is inhibited by 1 μM xestospongin C, a blocker of IP_3-stimulated Ca^{2+} mobilization from intracellular stores. 100 nM GnRH was applied for 5 minutes where indicated. Net responses were quantified (CPP) for 10 minutes after the start of GnRH application. Cells were prepared from sexually regressed goldfish. *Star* indicates a response significantly different from control (using students t-test; n=4). GTH-II data are in Chapter 5.

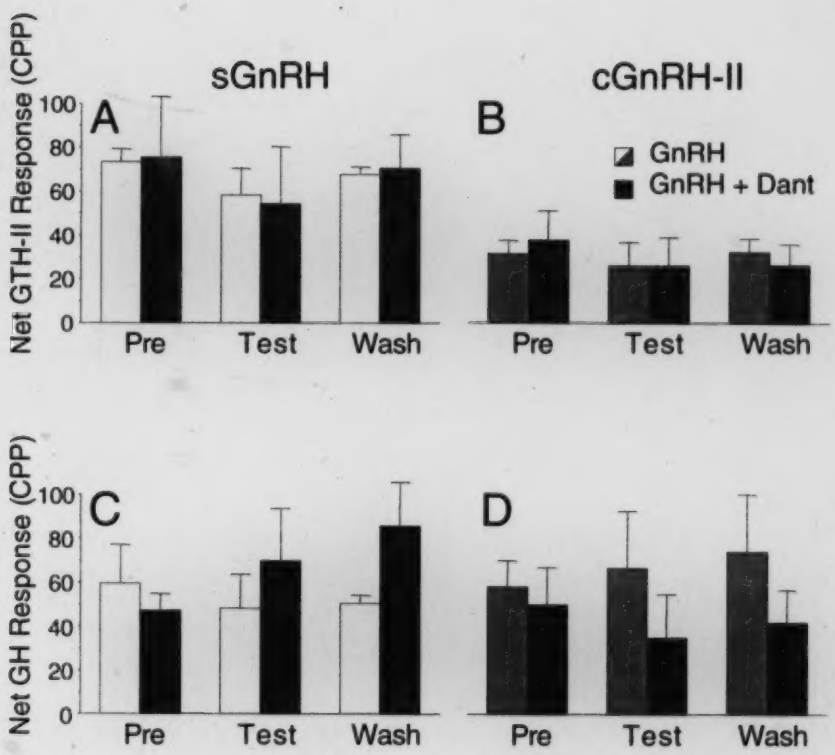

Figure 8.4. Hormone release evoked by sGnRH or cGnRH-II is not sensitive to 50 μM dantrolene (30 minute pretreatment), a skeletal RyR1 inhibitor. Bars represent GTH-II (A,B) or GH (C,D) responses to 100 nM GnRH, quantified from cell column perifusion experiments, as in Figure 2 (n=4). Cells were prepared from sexually regressed goldfish.

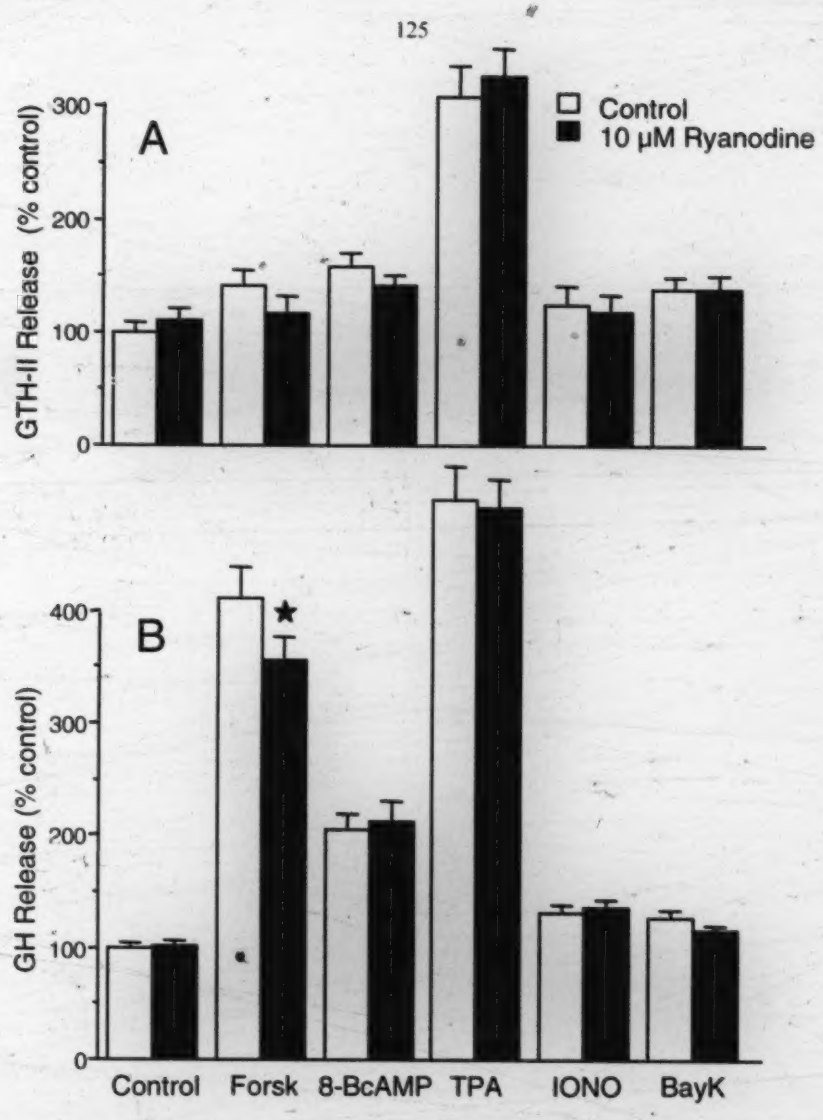

Figure 8.5. Effects of ryanodine on GTH-II (A) or GH (B) release in response to activators of signalling cascades previously implicated in the control of secretion from gonadotropes and somatotropes in goldfish. Cells were incubated for 2 hours with 10 µM forskolin, 1 mM 8-bromo-cyclic AMP, 10 nM TPA, 10 µM ionomycin or 10 µM BayK 8644 in the absence (*open bars*) or presence (*black bars*) of 10 µM ryanodine. Data from cell preparations from both sexually regressed and mature fish were similar and are therefore pooled (n=16). Star denotes a significant difference between ryanodine treated and non-treated cells.

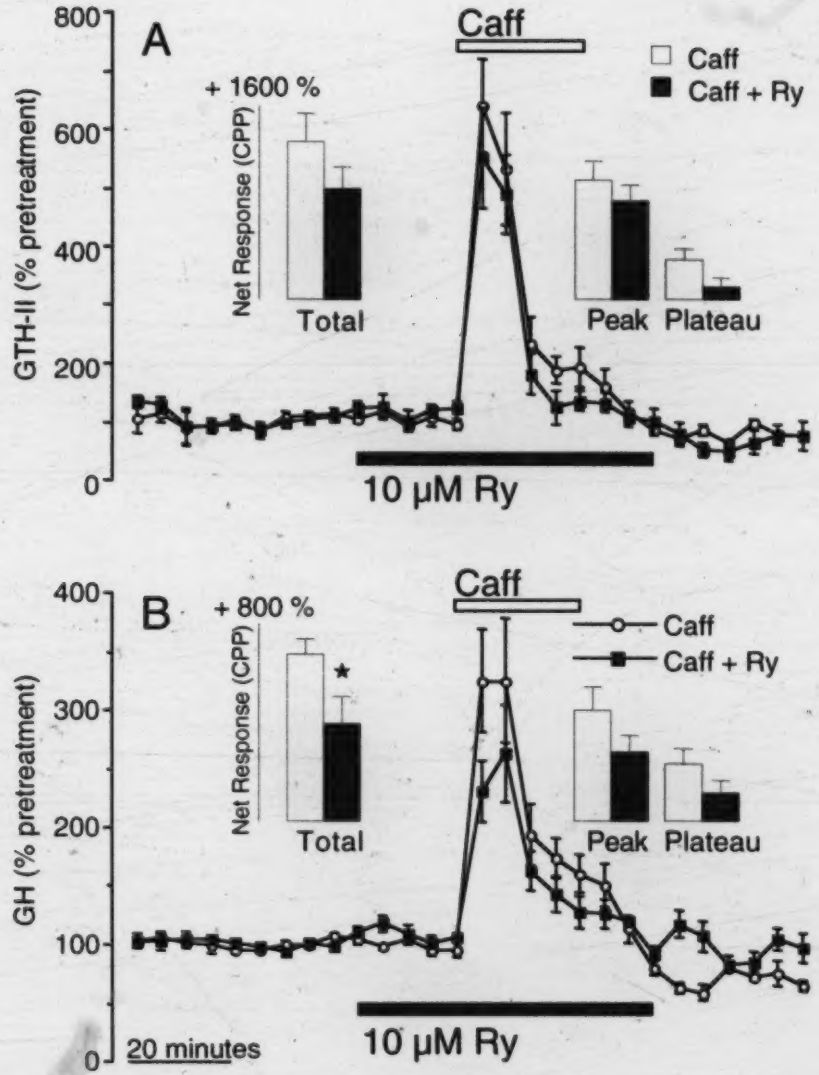

Figure 8.6. Effects of 10 µM ryanodine (*black horizontal bars*) on GTH-II (A) or GH (B) release responses evoked by 10 mM caffeine (*open horizontal bars*). Quantified hormone release responses to caffeine are shown in the *Insets*. Data from cell preparations from both regressed and mature fish were similar and are therefore pooled. *Star* indicates a response significantly different from control (n=8).

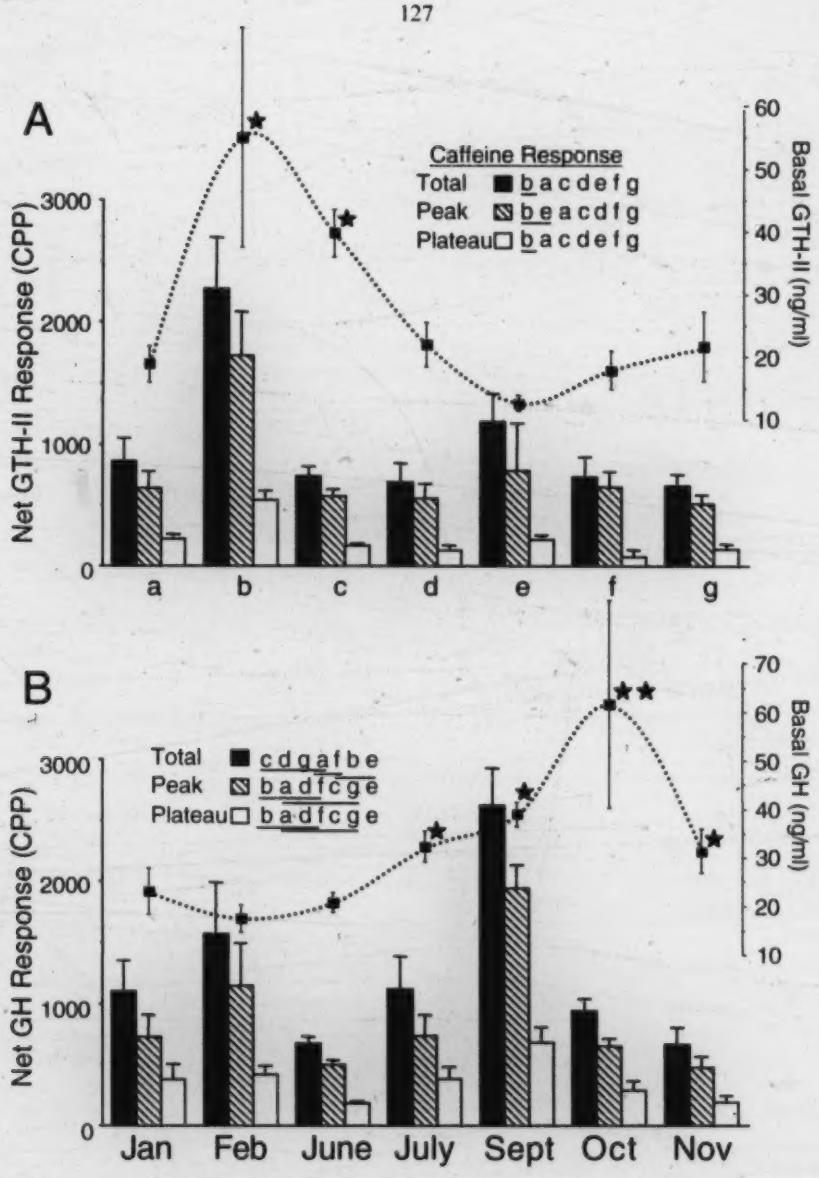
A
Caffeine Response
Total ■ b a c d e f g
Peak ▨ b e a c d f g
Plateau□ b a c d e f g
Net GTH-II Response (CPP)
Basal GTH-II (ng/ml)
3000
2000
1000
0
60
50
40
30
20
10
a
b
c
d
e
f
g
B
Total ■ c d g a f b e
Peak ▨ b a d f c g e
Plateau□ b a d f c g e
Net GH Response (CPP)
Basal GH (ng/ml)
3000
2000
1000
0
70
60
50
40
30
20
10
Jan
Feb
June
July
Sept
Oct
Nov

Figure 8.7. Seasonal variation in the amplitude of 10 mM caffeine-stimulated GTH-II (A) and GH (B) release. Responses were quantified from perifusion experiments similar to those reported in Figure 6 in exactly the same manner. *Insets* show the basal hormone release from these columns (which is representative of pre-normalization basal hormone release in all other figures). Data from a total of 85 columns are shown (n>6 for each month). Months are coded alphabetically. Letters with the same underscore represent caffeine response values that are not statistically different. Basal hormone values with which significantly different from each other do not share the same number of stars.

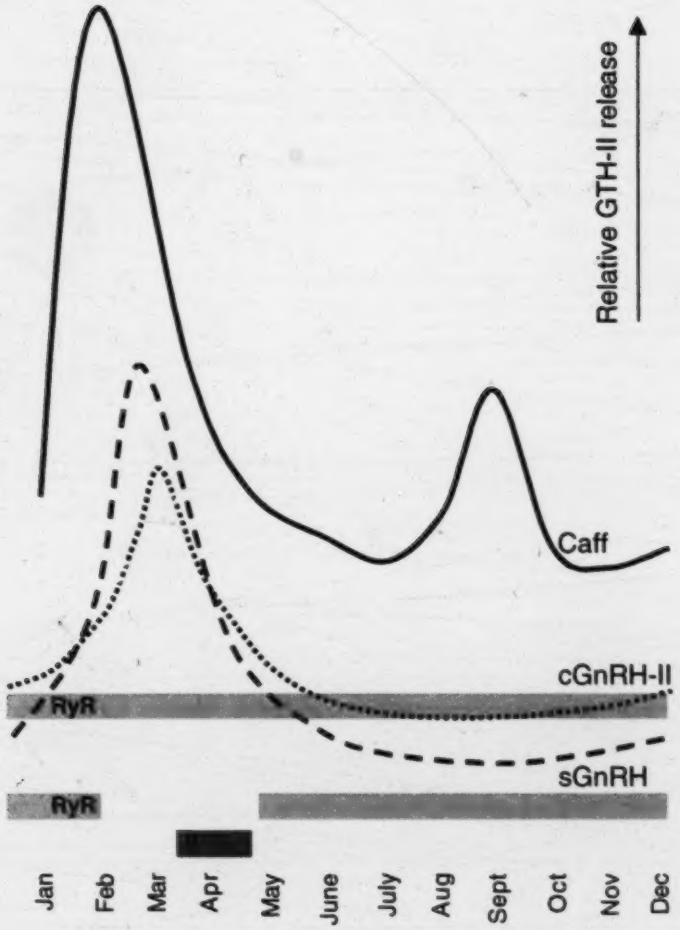

Figure 8.8. Limited, hypothetical model of seasonality of intracellular Ca^{2+} store involvement in goldfish gonadotropes. The caffeine timeline (*solid line*) is taken from Fig. 8.7. sGnRH (*large dashes*) and cGnRH-II (*short dashes*) timelines are approximated from data in this thesis, Lo and Chang, 1998, and unpublished data. Continuous bars indicating RyR involvement in GnRH-stimulated GTH-II release are based on the limited data from experiments done at several timepoint during the year, but should not be considered comprehensive. The bar indicating IP_3R involvement is based on one set of experiments in April and, therefore, it is not known whether it can be extended throughout the year.

Chapter 9.

Ca^{2+} buffering activity of mitochondria is strongly coupled to growth hormone secretion

Understanding the homeostatic interactions between intracellular Ca^{2+} stores is important if we are to comprehend how cells generate a diversity of Ca^{2+} signals that control multiple Ca^{2+}-dependent processes (reviewed in Johnson and Chang, 2000a). A critical role for mitochondria in the maintenance of cellular Ca^{2+} homeostasis is becoming accepted for many cell types, including mammalian pituitary cells (Duchen, 1999; Hehl et al., 1996; Herrington et al., 1996). Mitochondria are capable of rapidly and transiently buffering large quantities of Ca^{2+} by virtue of a Ca^{2+} uniporter driven by the mitochondrial proton gradient (Duchen, 1999; Sparagna et al., 1995). Ca^{2+} is released from intact mitochondria primarily by Na^{+}/ Ca^{2+} exchange (Duchen, 1999). It is thought that mitochondria act as a Ca^{2+} shuttle by subsequently releasing sequestered Ca^{2+} into other Ca^{2+}-storing organelles.

It is believed that mitochondrial Ca^{2+} uniporters and ER Ca^{2+} release channels reciprocally interact to regulate metabolic processes in the mitochondria, as well as the generation of Ca^{2+} signals from ER Ca^{2+} channels (reviewed in Chapter 1). This situation arises because mitochondria sense and buffer high-amplitude, localized Ca^{2+} release events produced in their immediate vicinity by Ca^{2+}-release channels, such as IP_3 receptors (Rizzuto et al., 1993; Rizzuto et al., 1998; Simpson et al., 1997, Csordás et al., 1999, Duchen, 1999). Conversely, mitochondrial Ca^{2+} uptake may act as positive or negative regulators of IP_3 receptor-mediated Ca^{2+} signals by modulating the open probability of Ca^{2+}-sensitive IP_3 receptor channels (Jouaville et al., 1995; Hajnóczky et al., 1999, Csordás et al., 1999, Duchen, 1999). Currently, less is known about the potential functional interactions between the mitochondria and IP_3-insensitive Ca^{2+} stores.

Pharmacological treatments that prevent mitochondria from sequestering Ca^{2+} by collapsing the proton gradient have been used to study the role of mitochondrial Ca^{2+} uptake in shaping the spatial and temporal characteristics of agonist-evoked Ca^{2+} signals (Tinel et al., 1999; Hajnóczky et al., 1999) and Ca^{2+} store refilling (Hoth et al., 1997). However, the roles of mitochondrial Ca^{2+} handling in Ca^{2+}-dependent functions, such as hormone release, are poorly understood. The relationship between mitochondria and hormone secretion has predominantly been studied in pancreatic β-cells, where mitochondrial uncoupling may affect hormone secretion by modulating metabolism and ATP-sensitive membrane excitability (Misler et al., 1992, Rustenbeck et al., 1997). However, this scheme may not be representative of all cell types. Reports on the effects of mitochondrial Ca^{2+} buffering on unstimulated hormone secretion in other cell types are lacking.

Results from previous chapters in this thesis have revealed an important role for intracellular Ca^{2+} stores in regulating $[Ca^{2+}]_c$, basal GH release and GnRH-stimulated GH release (Chapters 7,8). In this chapter, I investigate the involvement of mitochondrial Ca^{2+} buffering, and its interactions with other Ca^{2+}-dependent signal transduction cascades, in goldfish somatotropes. Ruthenium red

(RR) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which inhibit the Ca^{2+} uniporter by direct and indirect mechanisms, respectively (Moore, 1971, Duchen, 1999; Hehl et al., 1996, Jou et al., 1996, Tang et al., 1997, Jouaville et al., 1995, Zhu et al., 1999), were used to probe mitochondrial involvement in basal GH secretion and Ca^{2+} homeostasis. Using this approach, data is presented that suggests that inhibition of mitochondrial Ca^{2+} uptake evokes GH release through the generation of relatively small amplitude Ca^{2+} signals. Furthermore, we describe a novel, physiologically relevant link between the mitochondria and intracellular Ca^{2+} store sensitive to caffeine and sGnRH, but not cGnRH-II.

Results

Inhibitors of mitochondrial Ca^{2+} uptake elevate $[Ca^{2+}]_c$

Ca^{2+} imaging was used to examine the effects of CCCP and RR on $[Ca^{2+}]_c$ in goldfish somatotropes. Goldfish somatotropes were generally quiescent. However, spontaneous non-periodic fluctuations of approximately 100 nM can be measured in a sub-population comprising approximately 20% of somatotropes (see also Chapter 7). In the present study, only cells with a stable resting $[Ca^{2+}]_c$ were used. Application of 10 µM RR or 10 µM CCCP resulted in a rapid and reversible increase in $[Ca^{2+}]_c$ (Figs. 9.1, 9.2). Multiple Ca^{2+} signals could be evoked in the same cell by CCCP (25 minutes between challenges; n=2; not shown). Ca^{2+} signals generated by inhibition of the mitochondrial uniporter were relatively small in amplitude (CCCP: 37 ± 8 nM, 51 ± 7% above baseline, n=9; RR: 83 ± 32 nM, 119 ± 59 % above baseline, n=5). The waveforms of the Ca^{2+} signals induced by mitochondrial inhibition differ considerably from those evoked by GnRH (Chapter 7) or 30 mM KCl (Chapter 7 and Figs. 9.1, 9.2). For example, CCCP- and RR-generated Ca^{2+} signals do not show robust decay during continuous drug exposure.

The plateau-like temporal profile of the CCCP- and RR-generated Ca^{2+} signals may offer clues as to the mechanism of their origin. The Ca^{2+} signals often began with one or two transient fluctuations followed by a sustained period of elevated $[Ca^{2+}]_c$ (Figs. 9.1, 9.2). These recordings support a model whereby, the inhibition of mitochondrial Ca^{2+} uptake increases the probability of spontaneous Ca^{2+} events developing into measurable Ca^{2+} signals. Therefore, it is possible that mitochondria tonically buffer intracellular Ca^{2+}. These data also indicate that somatotropes can generate multiple Ca^{2+} signals with distinct temporal profiles.

Spatial analysis of Ca^{2+} signals generated by inhibition of mitochondrial Ca^{2+} buffering

Next, the spatial and temporal characteristics of Ca^{2+} signals generated by the inhibition of mitochondrial Ca^{2+} uptake were examined. At rest, goldfish somatotropes exhibit standing Ca^{2+} gradients, where $[Ca^{2+}]_c$ is higher in the region of the cell located away from the nucleus (Fig. 9.1B, n=11; see also Chapter 3). These gradients were consistently preserved and augmented during CCCP- and RR-generated Ca^{2+} elevations that were otherwise uniform around the non-nuclear regions of the cell. In other words, CCCP and RR generated Ca^{2+} signals that were predominantly localized opposite the nucleus and evoked Ca^{2+} increases

from the nuclear region were relatively smaller in amplitude (CCCP: Fig. 9.1C; RR data not shown). These features were not seen during subsequent KCl-stimulation in the same cell. The onset of the KCl-evoked Ca^{2+} signal was associated with a reversible collapse or a dramatic change in direction of the basal Ca^{2+} gradient (Fig. 9.1C).

Inhibitors of mitochondrial Ca^{2+} uptake stimulate GH release

Both CCCP and RR evoked significant GH release from cultured goldfish pituitary cells under both static incubation and cell column perifusion conditions (Figs. 9.3, 9.4). Although the observation that both agents similarly increased basal GH secretion strongly implicated mitochondrial Ca^{2+} uptake as the shared molecular target, other experiments were also performed to confirm their mode of action. Ryanodine receptors are an alternate target for RR in some cell types (Ehrlich 1994; Sundaresan et al., 1997). However, 10 μM ryanodine neither mimicked the effects of RR on GH release or $[Ca^{2+}]_c$ (data not shown), nor exerted any significant effects on RR-stimulated GH release (Fig. 9.4C). Although CCCP may modify pH_i in some cell types (Sidky and Bainbridge, 1997), the observation that CCCP-stimulated GH release could be completely abolished by pre-incubating cultures with 50 μM BAPTA-AM strongly implicates changes in $[Ca^{2+}]_c$ in its mechanism of action (Fig. 9.3D). The results of these pharmacological manipulations, taken together with the similarities in the kinetics of the hormone release responses and Ca^{2+} signals induced by CCCP and RR, suggest that these two inhibitors exert their secretagogue effects by altering Ca^{2+} homeostasis at the level of the mitochondrial Ca^{2+} uniporter in goldfish somatotropes.

Functional relationships between mitochondria and various Ca^{2+} sources

Having obtained data suggesting that the mitochondria are tonically active in buffering spontaneous $[Ca^{2+}]_c$ elevations in a circuit that is highly coupled to hormone secretion, we sought to elucidate the source of this basal leak of Ca^{2+} using GH release as an indicator of physiologically relevant interactions between the mitochondrial Ca^{2+} uniporter and various possible Ca^{2+}-release mechanisms. The possible interaction with Ca^{2+} entry through plasma membrane VGCCs was examined first. It is unlikely that the buffered Ca^{2+} arises from the influx of extracellular Ca^{2+} through these channels since RR did not potentiate GH secretion evoked by the VGCC activator BayK 8644, in static incubation experiments (Fig. 9.5).

Next, the possibility that Ca^{2+} buffering mediated by mitochondrial uptake is coupled to one or more of the multiple intracellular Ca^{2+} stores found in goldfish somatotropes was evaluated. We considered whether mitochondria interact with Tg-sensitive Ca^{2+} stores, which do not normally couple to hormone release. We reasoned that if there was a physiologically-relevant link between mitochondria and Tg-sensitive Ca^{2+} stores, then inhibition of mitochondrial Ca^{2+} buffering may uncover Tg-stimulated GH release by modulating dynamics of Tg-evoked Ca^{2+} signals, perhaps by permitting them to propagate to specific cellular regions (Tinel et al., 1999). However, the inhibition of mitochondrial Ca^{2+}

buffering with CCCP failed to convert Tg into a secretagogue treatment (n=6, data not shown) suggesting that these two Ca^{2+} pools do not interact with respect to exocytosis. In contrast, Ca^{2+} release from caffeine-sensitive stores appears to be coupled to tonic Ca^{2+} buffering of mitochondria, in a way that is important for the regulation of GH release. CCCP may have potentiated the decay (plateau-like) portion of caffeine-stimulated GH release (Fig. 9.6A). Similarly, a substimulatory dose of RR (10 nM) potentiated caffeine-evoked GH secretion in 2-hour static incubation experiments (Fig. 9.6B). Together, these data demonstrate that the caffeine/GnRH-sensitive intracellular Ca^{2+} stores and mitochondria are coupled in a functionally important subcellular Ca^{2+} circuit.

With the knowledge that several Ca^{2+} stores may interact and compensate for each other, the effects of blocking mitochondrial Ca^{2+} uptake on caffeine-stimulated GH release was examined in the presence of 100 μM ryanodine to eliminate Ca^{2+} cycling through an additional intracellular Ca^{2+} pool. Surprisingly, this manipulation evoked an immediate, massive potentiation of all phases of caffeine-stimulated GH release (10 fold higher than has been observed in this and pervious studies in this model system; see Chapters 7, 8) that was completely reversible (Fig. 9.6C). This greatly enhanced GH release response was dependent on the presence of both RR and ryanodine. When tested alone in perifusion studies, RR produced only a minor increase and ryanodine a minor decrease of caffeine-stimulated GH release (n=4 RR data not shown; n=10 ryanodine Chapters 7,8). Moreover, caffeine-stimulated GTH-II release from the same experiments was not potentiated, indicating that the augmentation of GH release was specific. In addition, this dichotomy illustrated important functional differences between the Ca^{2+} signalling systems of these two cell types. These data can be interpreted as the enhanced coupling of mitochondrial Ca^{2+} uptake with caffeine-sensitive Ca^{2+} stores when Ca^{2+} is not allowed to cycle through a third Ca^{2+} pool.

Finally, we examined whether Ca^{2+} signalling cascades of endogenous agonists, sGnRH and cGnRH-II, are involved in the mitochondrial Ca^{2+} handling circuit. The experiments with caffeine, which seems to target the GnRH-sensitive Ca^{2+} stores, suggested that there could be an interaction. Surprisingly, sGnRH, but not cGnRH-II, was significantly potentiated in the presence of RR (Fig. 9.7). These data indicate that mitochondrial Ca^{2+} uptake can selectively modulate signalling, in an agonist-specific manner. Furthermore, this finding is novel evidence supporting the idea that the two GnRHs may act through dissimilar signalling pathways in goldfish somatotropes.

Discussion

Involvement of mitochondria in Ca^{2+} homeostasis and GH release

Over the past several years, there has been renewed interest in the role of mitochondrial Ca^{2+} buffering during physiological stimulation of many cell types (reviewed in Duchen, 1999). Data presented herein show, for the first time, the functional consequence of mitochondrial Ca^{2+} uptake on $[Ca^{2+}]_c$ and hormone release under unstimulated conditions. In addition, the functional consequences of

interactions between mitochondria and multiple intracellular Ca^{2+} stores are revealed.

Results from Ca^{2+} imaging experiments with CCCP and RR strongly suggest that the mitochondrial Ca^{2+} uniporter plays a small, but physiologically significant, role in the Ca^{2+} homeostasis in unstimulated goldfish somatotropes. The magnitude and timecourse of the Ca^{2+} signals evoked by CCCP and RR treatment are similar to those observed in other cell types, including rat pituitary cells (Hehl et al., 1996). However, parallel investigations on the role of mitochondria in basal hormone or neurotransmitter release are lacking. In the present Chapter, the demonstration of rapid and sustained hormone release responses to both CCCP and RR provides strong evidence that Ca^{2+} buffering by the mitochondrial Ca^{2+} uniporter regulates basal GH release in cultured goldfish somatotropes. Consistent with the hypothesis that mitochondria can be involved in basal hormone release, Giovannucci and co-workers (1999) recently demonstrated that mitochondrial Ca^{2+} uptake may regulate the recruitment of secretory granules into a releasable pool in bovine chromaffin cells. Unlike our results, mitochondrial inhibition did not affect resting $[Ca^{2+}]_c$ or basal exocytosis in chromaffin cells, although a significant enhancement of evoked Ca^{2+} signalling and exocytosis was observed (Giovannucci et al., 1999; Montero et al., 2000).

It is unlikely that other actions resulting from inhibition of the mitochondrial Ca^{2+} uniporter, such as reducing $[ATP]_c$, are responsible for the rapid and sustained elevation in $[Ca^{2+}]_c$ and GH release. Reasons for this argument can be summarized as follows: 1) a significant reduction of ATP (which is required for operation of the secretory pathway) would be expected to reduce secretion; 2) subsequent caffeine- and GnRH-stimulated secretion, which would be expected to possess ATP-dependent components, is not reduced by pre-exposure to CCCP/RR; 3) although ATP depletion could modify Ca^{2+} homeostasis by inhibiting SERCA, direct inhibition of SERCA with Tg does not mimic either Ca^{2+} signals or the hormone release evoked by CCCP/RR (see Chapters 7, 12); 4) Ca^{2+} signals and GH secretion begin immediately, suggesting that they are not due to the slow depletion of metabolites; 5) CCCP-stimulated GH release is abolished by BAPTA, indicating that its effects are mediated through Ca^{2+} transients. It is also noteworthy that basal GH release in 2-hour static incubation experiments is not affected by either diazoxide or glibenclamide, an activator and blocker of ATP-sensitive K^+ channels, respectively (WK Yunker and JP Chang, unpublished results). Perhaps it is telling that it is in studies where cells were permeabilized or the cytoplasm dialyzed (i.e. whole cell patch clamp) that investigators have indicated a requirement for inclusion of ATP in the pipette to control for the effects of mitochondrial uncoupling on cellular energetics (Hehl et al., 1996; Herrington et al., 1996). Thus, in our experiments on intact somatotropes, the effects of CCCP on $[Ca^{2+}]_c$ and GH release are likely due to an inhibition of mitochondrial Ca^{2+} uptake through the Ca^{2+} uniporter.

The observation that CCCP and RR exert very similar effects on both $[Ca^{2+}]_c$ and GH release strongly argues that they act on the same cellular system. In addition, CCCP and RR responses were not additive; subsequent applications of 10 μM RR were unable to evoke an additional increase in $[Ca^{2+}]_c$ when cells

had been pre-exposed to 10 µM CCCP (n=2, not shown). The similarity to CCCP action and presence of RR-stimulated Ca^{2+} signals also strongly suggest that RR does not act as a Ca^{2+}-independent regulator of secretion, as has been postulated for neuronal exocytosis by one group (Trudeau et al., 1996). Despite previous reports that RR is not membrane permeant, several investigators have found effects consistent with an intracellular site of action when RR was applied to bathing solutions of several cell types, including pituitary cells (e.g. Sundaresan et al., 1997; Jou et al., 1996; Zhu et al., 1999). This highly charged hexavalent cation is expected to have considerable affinity for negative charges on cation carriers and intracellular proteins, in addition to its actions as a membrane stain (Voelker and Smejtek, 1996). Since RR blocks the Ca^{2+} uniporter of isolated mitochondria at nanomolar concentrations (Moore, 1971; Sparagna et al., 1995) it is likely that the high concentrations of RR used in the present study may allow for adequate RR entry and activity. Although we have not measured mitochondrial Ca^{2+} uptake directly, these arguments strongly suggest that the Ca^{2+} signals and GH release measured in the present study are due to specific inhibition of the Ca^{2+} uniporter.

Mitochondrial uncoupling does not lead to a transient $[Ca^{2+}]_c$ rise in all cell types (Duchen, 1999). The reason for this difference is not clear, but it may reflect resting Ca^{2+} levels in mitochondria (Babcock et al., 1997). In neurons, the mitochondria must be pre-loaded with a KCl pre-pulse before CCCP can increase $[Ca^{2+}]_c$, suggesting that mitochondria are not active under basal conditions (Budd et al., 1995). It is unlikely that uncouplers released stored Ca^{2+} from mitochondria in our experiments on naïve cells. In all cell types where it has been examined *in situ*, normal levels of Ca^{2+} in mitochondria are similar to the cytosol ($[Ca^{2+}]_m$ = 80-200 nM; Babcock et al., 1997 and references therein). With this concentration gradient, even if the mitochondrial membrane potential collapsed to zero, only a small amount of Ca^{2+} would be released from mitochondria. Whether mitochondrial Ca^{2+} buffering is a continuous processes (e.g. goldfish somatotropes) or is recruited only during stimulation (e.g. neurons) may reflect different basal Ca^{2+} signalling systems or ER-mitochondrial proximity between different cell types.

Characterization of multiple spatial patterns and temporal waveforms of Ca^{2+} signals

Spatial and temporal features of Ca^{2+} signals can encode functionally important and specific information (see Chapter 1). In the present study, Ca^{2+} signals generated by inhibiting mitochondrial Ca^{2+} uptake seemed to be restricted from the nucleus. During Ca^{2+} signals generated by CCCP and RR, the Ca^{2+} gradient is not only preserved but magnified, whereas it is reduced, abolished or reversed in KCl-stimulated Ca^{2+} signals. Whether this feature is important for the secretagogue efficiency of the CCCP- and RR-evoked Ca^{2+} signals remains to be determined. Although it is clear that this phenomenon is present in goldfish somatotropes, the mechanism by which these gradients are created, sustained and abolished also requires attention. Although the cytosolic and nuclear compartments are linked through nuclear pores, differential regulation of nuclear

and cytosolic Ca^{2+} has been demonstrated in many cell types (reviewed in Rogue and Malviya, 1999). From these "macroscopic" data, functional specificity of Ca^{2+} signals in goldfish somatotropes cannot be easily ascertained. It is likely that measurements of bulk cytosolic free [Ca^{2+}] misrepresent, in both space and time, the dynamics of the Ca^{2+} events that are important in deciding which specific cellular functions are triggered. Nevertheless, results from the present study, clearly reveal that distinct Ca^{2+} signals can be evoked in goldfish somatotropes. Such information on the spatial characteristics of measurable Ca^{2+} signals provides an important first step towards understanding the complexity of the submicroscopic Ca^{2+} signalling events that likely underlie the specific regulation of multiple Ca^{2+}-dependent cellular functions.

Specific functional interactions between Ca^{2+} stores

Goldfish somatotropes contain multiple intracellular Ca^{2+} stores and at least one mechanism for the influx of extracellular Ca^{2+}. In addition to the present results with Bay K 8644, other lines of evidence indicate that the influx of extracellular Ca^{2+} through VGCCs is not the source of the Ca^{2+} events buffered by mitochondria in this cell type, as was found to be the case in neurons (Svichar et al., 1997). The Ca^{2+} oscillations that are occasionally recorded from goldfish somatotropes likely originate from TMB-8-sensitive intracellular Ca^{2+} stores (Chapter 7). The ultrastructural identity of these Ca^{2+} stores in goldfish somatotropes is not known, but the ER has been implicated as a source of Ca^{2+} buffered by mitochondria in other cell types. Consistent with this possibility, examination of previously published and unpublished electron micrographs of goldfish somatotropes revealed that mitochondria are always located next to ER, and are not generally found near the plasma membrane (Leatherland, 1971, JD Johnson, JP Chang and H Cook, unpublished observations). Proximity to local Ca^{2+} release channels and associated high-amplitude Ca^{2+} microdomains are important factors in determining the relative contribution of the low affinity Ca^{2+} uptake of the mitochondrial uniporter (Duchen, 1999). Results indicating that Ca^{2+} levels adjacent to the mitochondria can reach these levels without concomitant global Ca^{2+} signals have been presented for several cell types (Rizzuto et al., 1993; Rizzuto et al., 1998).

Results from the chapter show that mitochondria interact with a novel class of caffeine-sensitive Ca^{2+} stores to regulate GH release, and that this interaction is accentuated when Ca^{2+} cycling through ryanodine-sensitive stores is blocked. Interestingly, although caffeine-sensitive Ca^{2+} stores participate in the GH-releasing action of both GnRHs (Chapter 7), the signalling cascades of sGnRH and cGnRH-II can apparently be distinguished by their differential involvement of mitochondrial Ca^{2+} uptake. Thus far, the major differences between the signal transduction of the GnRHs is the selective involvement of IP_3R in sGnRH signalling, and the selective involvement of RyR in cGnRH-II signalling, during sexually regression (Chapters 5, 8). It is possible that the differential involvement of these Ca^{2+} signalling systems may underly the selective modulation of sGnRH-stimulated GH release by RR treatment. Regardless of the exact mechanisms, it is clear that complex and specific

interactions occur among multiple intracellular Ca^{2+} stores in goldfish somatotropes.

Although recent studies have demonstrated that mitochondria can buffer Ca^{2+} microdomains originating from RyR (Szalai et al., 2000, Nasser et al., 2000), the majority of the literature has focus on the intimate physical and functional connections between the IP_3R and Ca^{2+} transport into the mitochondria (reviewed in Duchen 1999). The molecular mechanisms that maintain the specific association of mitochondrial Ca^{2+} uniporters with Ca^{2+} release channels remains to be determined. Both IP_3R and RyR have large cytosolic domains which could mediate protein-protein interactions with specific scaffolding proteins or components of the cytoskeleton. The present results provide a good starting point for studies on how mitochondria may interact with specific Ca^{2+} release channels in cell types which contain multiple Ca^{2+} stores.

Although an interaction between mitochondrial Ca^{2+} uptake and Tg-sensitive Ca^{2+} stores was not observed, it is still likely that SERCA play a prominent role in the regulation of somatotrope homeostasis. The fact that cellular Ca^{2+} homeostasis is maintained at a stable elevated level during treatments that block mitochondrial uptake indicates that additional Ca^{2+}-clearing mechanisms, such as Tg-sensitive SERCA pumps, are important. The involvement of other transport mechanisms, such as the plasma membrane Ca^{2+}ATPase and Na^+/Ca^{2+} exchange, also remains to be investigated. Undoubtedly, the maintenance of $[Ca^{2+}]_c$ in the range required for cell function and survival utilizes several, partially redundant mechanisms.

Physiological significance and summary

Results presented in this chapter indicate that mitochondria are involved in regulating basal and agonist-evoked GH secretion and play a constitutive role in setting resting $[Ca^{2+}]_c$ in goldfish somatotropes. Based on the present findings, a working hypothesis is formulated (Fig. 9.8). Inhibition of mitochondrial Ca^{2+} uptake would lead to a failure to buffer spontaneous Ca^{2+} release from Tg-insensitive stores. This increases the probability of initiating global Ca^{2+} signals from random spontaneous events. These Ca^{2+} signals may have special kinetic properties or follow a unique spatial route that is more highly coupled to exocytosis.

Multiple neuroendocrine regulators, with distinct signal transduction pathways have been shown to regulate GH release in goldfish (Chang et al., 2000; Peter and Chang, 1999). Many of the pathways used by these agonists, including cAMP and protein kinase C, are Ca^{2+}-sensitive to varying degrees. The degree of involvement of mitochondrial Ca^{2+} handling in these signalling cascades remains to be investigated. Previously, we have also shown that agonist-evoked GH secretion is Na^+-dependent, in a manner that does not involve voltage-gated Na^+ channels (Van Goor et al., 1997). Experiments on T-lymphocytes by Hoth and coworkers (1997) demonstrated that Na^+ depletion impairs the Ca^{2+} cycling through the mitochondria and the refilling of agonist-sensitive Ca^{2+} stores. The idea that Na^+-depletion targets cultured goldfish somatotropes through the inhibition of Na^+ efflux from mitochondria and the subsequent interference with

mitochondrial regulation of intracellular Ca^{2+} homeostasis, is an interesting, and hitherto untested, hypothesis.

In addition to the regulation of hormone release, the constitutive Ca^{2+} buffering activity may have other physiological consequences. Since the mitochondrial proton gradient drives both the production of ATP and the sequestration of Ca^{2+}, bursts in ATP synthesis would occur at the expense of Ca^{2+} homeostasis. More Ca^{2+} microdomains would escape mitochondrial uptake and combine to form Ca^{2+} transients that might lead to increased exocytosis representing a novel mechanism for coupling metabolism and exocytosis.

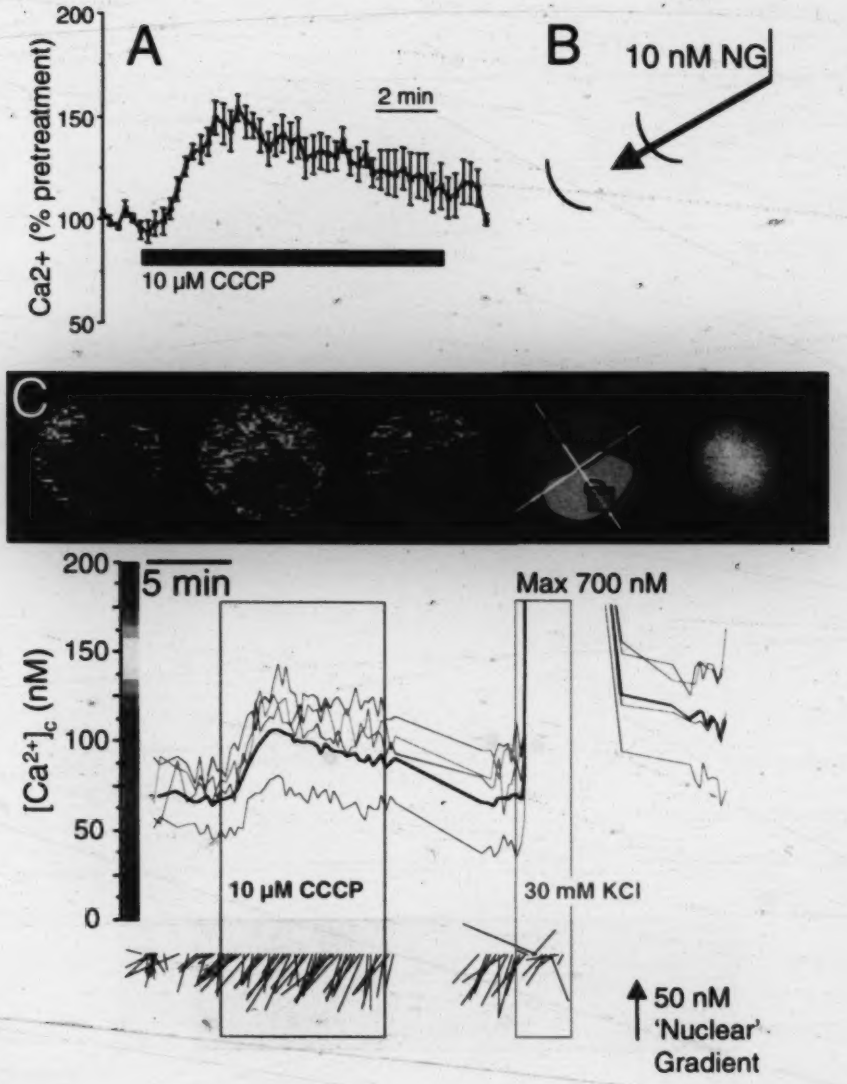
A
B
10 nM NG
2 min
Ca2+ (% pretreatment)
200
150
100
50
10 µM CCCP
C
5 min
Max 700 nM
[Ca2+]c (nM)
200
150
100
50
0
10 µM CCCP
30 mM KCl
50 nM
'Nuclear'
Gradient

Figure 9.1. Spatial and temporal characteristics of Ca^{2+} signals resulting from inhibition of mitochondrial Ca^{2+} uptake. A) Pooled timecourse of 10 μM CCCP-evoked Ca^{2+} signals in identified somatotropes (n = 9 independent cultures). B) The arrow represents the average standing Ca^{2+} gradient across the focal plane of resting somatotropes (data from the same experiments as in part A). The arcs are error bars, with their distance from the point of the arrow representing the SEM of the magnitude of the gradient and the their width representing the SEM of the angle of the gradient. The vertical calibration bar represents a 10 nM Ca^{2+} gradient in the nuclear direction (NG). C) Spatial analysis of Ca^{2+} signals elicited by inhibiting mitochondrial Ca^{2+} uptake with 10 μM CCCP. The top panel (from the left) shows false colour images of an identified somatotrope before, during and after treatment with 10 μM CCCP. The color key to these images is beside the y-axis. To the right of the false color images is a drawing of the cell same showing the position of the ovoid nucleus that occupies approximately half of the cross-sectional area. This cartoon also shows the position of the multiregional traces in the lower panel (colour squares) and of the orthogonal axes used in the vector analysis (lines). At the far right is an image of the fluorescence emission of Fura-2 at 380 nm in the same cell, demonstrating that the gradient does not arise from an artifact of uneven dye loading. The next panel shows traces of $[Ca^{2+}]_i$ levels in several manually-selected subcellular areas. Colored lines match the colored squares in the cell cartoon, whereas the thick black line represents the whole cell average $[Ca^{2+}]_i$. Note the delay in onset and the lower amplitude of the CCCP-evoked Ca^{2+} signal in the nuclear region. A subsequent depolarization by application of 30 mM KCl generates a spatially and temporally distinct Ca^{2+} signal. A subsequent application of 30 mM KCl produced a similar reversible Ca^{2+} signal (not shown). The bottom panel shows vector analysis of the same images/points as in part B. Inhibition of mitochondrial Ca^{2+} uptake preserves and magnifies the cellular Ca^{2+} gradient, while depolarization reduces and alters its direction. The example shown is representative of 9 similarly analyzed cells from independent cultures (from the same experiments as in part A).

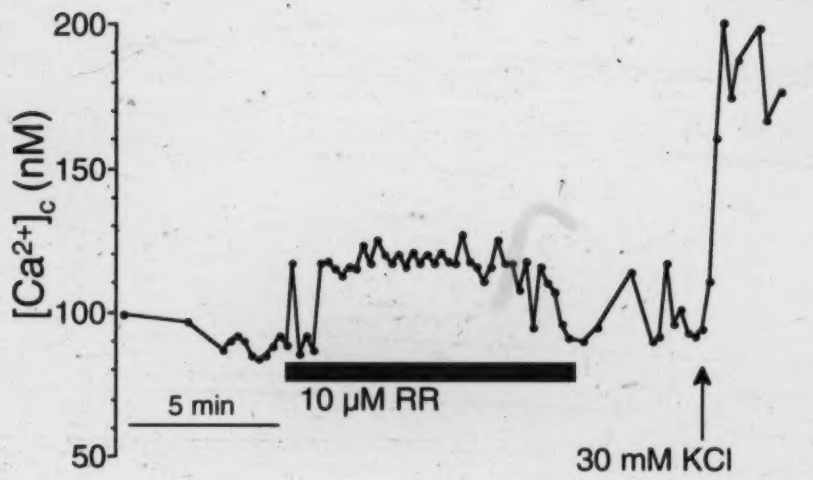

Figure 9.2. Inhibition of mitochondrial Ca^{2+} uptake with 10 µM RR evokes plateau like Ca^{2+} signals in identified somatotropes. Note the Ca^{2+} oscillation immediately after the application of RR. 30 mM KCl was applied as a positive control (*arrow* indicates the start of KCl treatment; KCl was not removed). The trace is representative of responses in cells from 5 independent cultures.

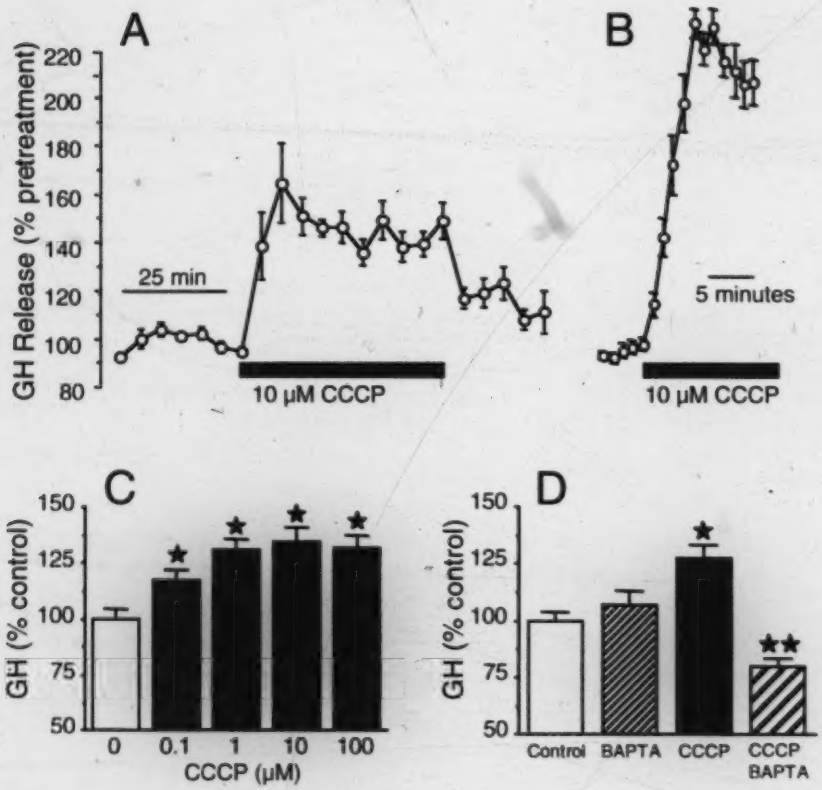

Figure 9.3. Inhibition of mitochondrial Ca^{2+} uptake with CCCP elicits GH secretion. A) Timecourse of 10 μM CCCP-evoked GH release from continuously-perifused cultured goldfish pituitary cells (n = 10). B) Analysis of the onset of 10 μM CCCP-stimulated GH release by collecting fast fractions (1 minute) from cell columns perifused as above (n = 12). C) Dose-response analysis of CCCP was carried out in 2-hour static incubation experiments (n = 15). D) Pre-incubation of cultures in 50 μM BAPTA-AM blocks the stimulatory effects of 10 μM CCCP in 2-hour static incubation indicating the Ca^{2+}-dependence of CCCP effects on GH release (n = 6). *Stars* indicate means that are significantly different from control. *Double star* indicates statistical difference as compared to CCCP-stimulated cultures.

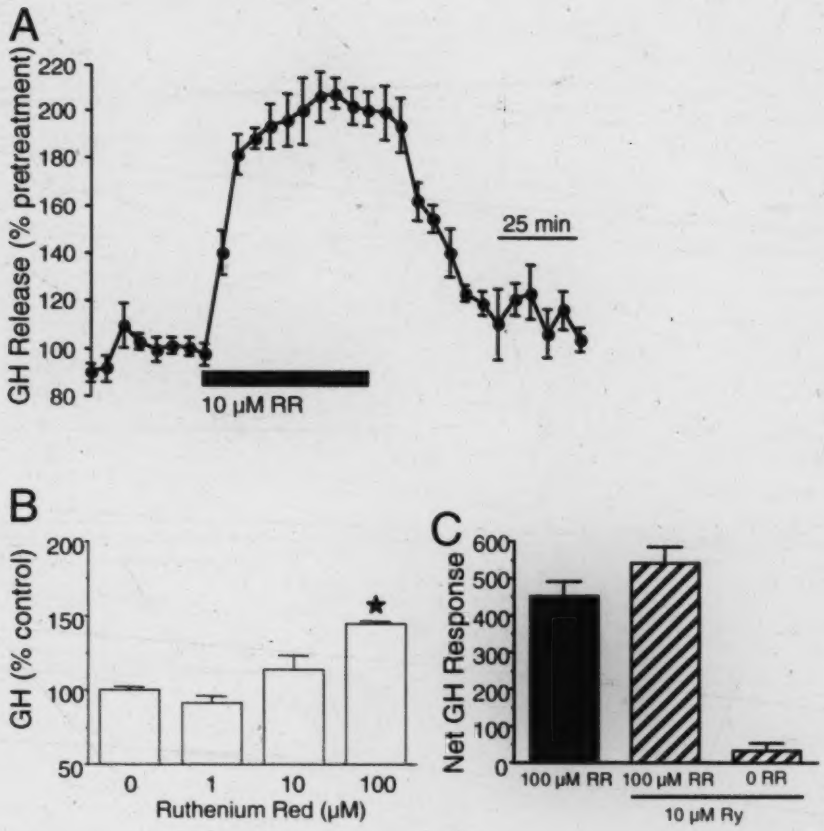

Figure 9.4. Direct inhibition of mitochondrial Ca^{2+} uptake with RR elicits GH secretion. A) Perifusion study of the timecourse of 10 µM RR-stimulated GH release indicates similarity to CCCP-evoked secretion (n = 8). B) Dose-dependence of RR-stimulated GH release in static incubation (n = 10). C) Dissimilarity of the responses to 100 µM RR and 100 µM ryanodine (Ry) indicate that RR does not evoke secretion by acting on ryanodine receptors (n = 4). *Star* indicates treatments significantly different from control.

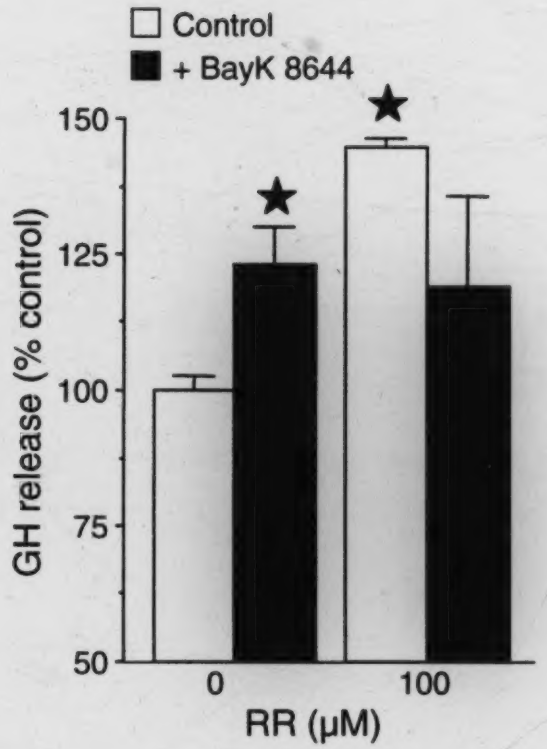

Figure 9.5. Inhibition of mitochondrial Ca^{2+} uptake does not potentiate GH release evoked by a VGCC agonist. Static incubation of dispersed pituitary cells with 10 μM BayK8644 does not affect 100 μM RR-stimulated GH release over 2 hours (n = 8). Similar results were seen with 10 μM RR (n=8). Stars indicate averages that are significantly different from control cultures.

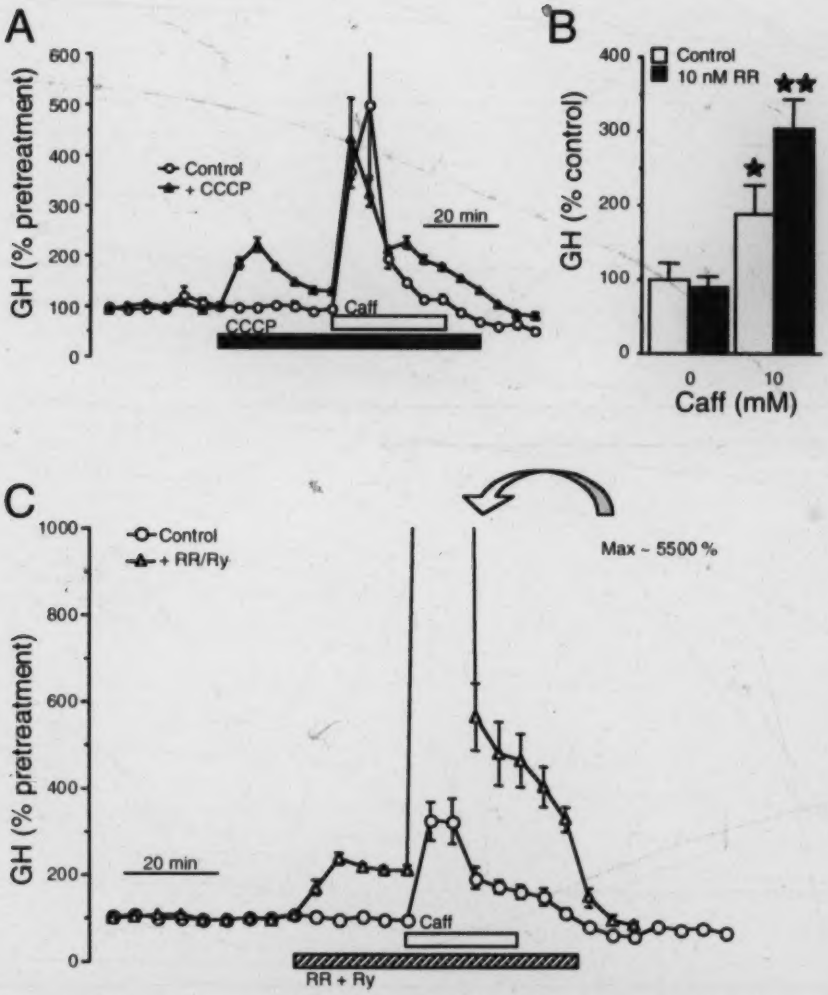

Figure 9.6. Functional consequences of interactions between mitochondrial and specific non-mitochondria Ca^{2+} stores. A) Treatment of cells with 10 μM CCCP potentiates the plateau phase of the 10 mM caffeine-stimulated GH release response in perifusion experiments (n = 4). B) GH release evoked by 10 mM caffeine is potentiated by a dose of RR that is not stimulatory on its own (10 nM) in 2-hour static incubation experiments (n = 8). *Single star* indicates significant difference from control. *Double star* indicates significant difference from control and cultures stimulated with caffeine alone. C) Simultaneous application of 100 μM RR and 100 μM Ry induces a massive potentiation of all phases of the 10 mM caffeine-stimulated GH release response in perifusion experiments (n = 4).

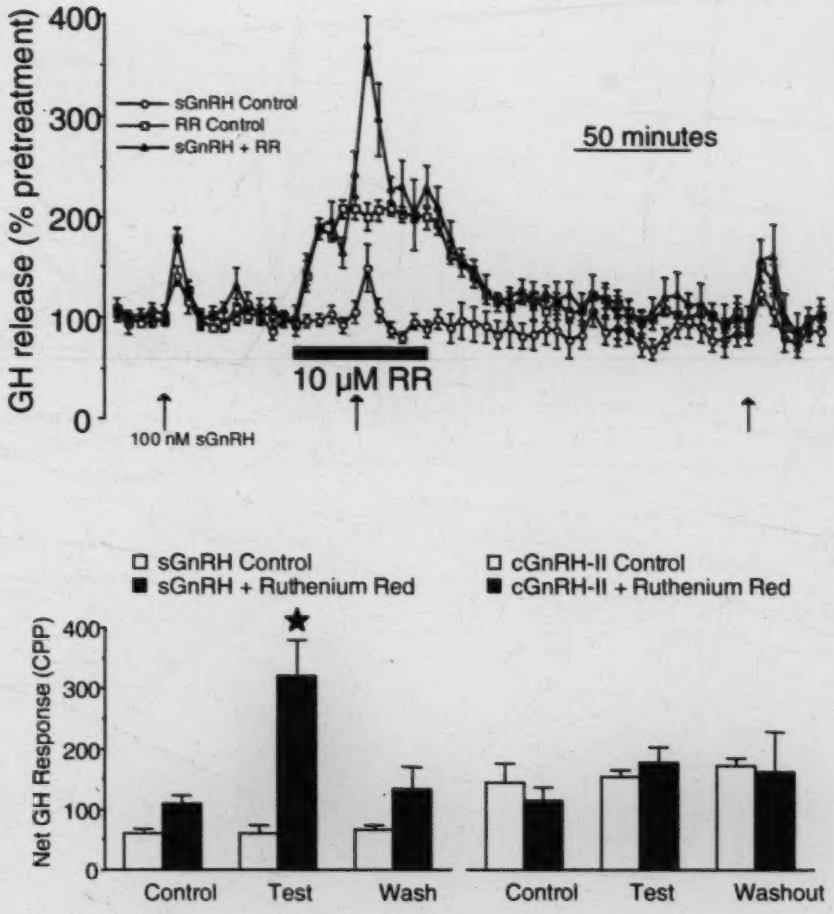

Figure 9.7. Inhibition of mitochondrial Ca^{2+} uptake selectively potentiates sGnRH-evoked GH release (A). Cell perifusion experiment showing the timecourse of GH release stimulated by 5-minute challenges with 100 nM sGnRH (*arrows*) in the presence and absence of 10 μM RR as indicated. B) 100 nM sGnRH- or 100 nM cGnRH-II-stimulated GH release responses are quantified (from the same experiments as in A) by integrated net GH released above baseline during the agonist-evoked response (20 minutes). Test pulse where sGnRH was given in the presence of RR is statistically different (*star*) from both sequential and parallel controls (n = 4).

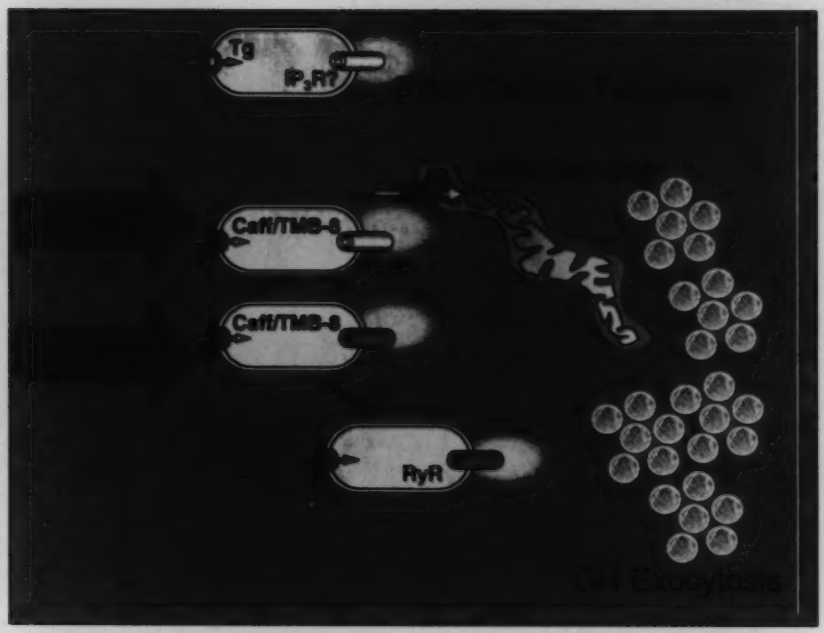

Figure 9.8. Working model of the putative intracellular Ca^{2+} signaling circuit in goldfish somatotropes, especially as it pertains to the roles of mitochondrial buffering in Ca^{2+} homeostasis and GH release. Submicroscopic Ca^{2+} gradients (microdomains) are depicted with yellow shading. Mitochondria act as a regulator of GH release through the activity of the Ca^{2+} uniporter which modulates the creation of global Ca^{2+} signals by Tg-insensitive Ca^{2+} stores, including the spontaneously-active agonist-sensitive Ca^{2+} pool. Ca^{2+} signals initiated by Ca^{2+} stores containing Tg-sensitive SERCA pumps, presumably IP_3-sensitive Ca^{2+} stores, are not involved in GH release. The Ca^{2+} uptake mechanisms of the independent GnRH/caffeine-sensitive and ryanodine-sensitive Ca^{2+} stores are not known. This cartoon illustrates the potential complexity of Ca^{2+} signaling circuitry in secretory cells, but may still be an oversimplification.

Chapter 10.

Rate of rise accounts for Ca^{2+} signal specificity in goldfish gonadotropes: Evidence for a novel form of Ca^{2+} signal coding

Ca^{2+} signals must be coded, to prevent unwanted intracellular cross-talk and maintain functional specificity. Cells employ both spatially and temporally specific Ca^{2+} signalling. Among temporal coding strategies, there is convincing evidence that the frequency (Pralong et al., 1994; Gu and Spitzer, 1995; Dolmetsch, 1998; De Koninck and Schulman, 1998), amplitude and duration (Dolmetsch, 1997; Hirabayashi et al., 1999) of Ca^{2+} signals can carry important information. Recently, it has been demonstrated that the rate of rise of an initial Ca^{2+} signal may dictate whether or not Ca^{2+}-induced Ca^{2+} release (CICR) will occur (Hernandez-Cruz, 1997), suggesting the possibility that the rate of rise may also confer function-specificity to Ca^{2+} signals.

In rat gonadotropes, the rate of exocytosis is correlated with the rapidity of the increase in $[Ca^{2+}]_c$ produced by 'uncaging' IP_3 at different speeds (Tse et al., 1997). In another study, the rate of the rising phase of mGnRH-evoked Ca^{2+} oscillations was correlated with exocytosis (Thomas and Waring, 1997). In goldfish gonadotropes, frequency and amplitude coding do not appear to be major factors in the control of hormone release, but the rate of rise of the Ca^{2+} signals generated by both sGnRH and cGnRH-II increases dose-dependently (Johnson et al., 1999; Chapter 4). We have also shown that cGnRH-II evokes Ca^{2+} signals with a significantly faster rate of rise than sGnRH, possibly due to the differential use of specific intracellular Ca^{2+} stores (Johnson et al., 2000; Chang et al., 1996; Chapters 4,5). It is likely that these and other differences in signal transduction of sGnRH and cGnRH-II play a role in their differential control of GTH-II gene expression under some conditions (Khakoo et al., 1994). Whether these agonist-specific Ca^{2+} signals differentially regulate acute GTH-II release cannot be ascertained from previous hormone release experiments with low temporal resolution.

Is the rate of rise of Ca^{2+} signals physiologically important? To answer this question, I have compared the GTH-II-releasing activity of several intracellular Ca^{2+}-mobilizing treatments that evoked Ca^{2+} signals of similar amplitude, but with different rates of rise, in single Fura-2-loaded goldfish gonadotropes. Specifically, slow-rising Ca^{2+} signals were generated by the passive leak of Ca^{2+} from intracellular stores revealed in the presence of Tg (Thastrup et al., 1990). Other Ca^{2+} signals were evoked by the rapid mobilization of pharmacologically distinct intracellular Ca^{2+} stores sensitive to sGnRH, cGnRH-II and caffeine. Results in this Chapter demonstrate that the effectiveness with which various treatments elicit GTH-II release in rapid-fraction perifusion studies appears to be related to the rate of rise, and not the amplitude, of global Ca^{2+} signals.

Results

Temporal comparison of Ca^{2+} signals

In order to test the hypothesis that rate of rise is an important feature of Ca^{2+} signals in gonadotropes, we systematically searched for treatments which generated Ca^{2+} signals with a similar amplitude, similar cellular location, but with different rate kinetics. An important part of this strategy was finding a treatment that evoked very slow rising Ca^{2+} signals. Application of 2 μM Tg generated slow-rising Ca^{2+} signals in identified gonadotropes (Fig. 10.1 A,B). All cells responded significantly to Tg according to the criteria defined in the Chapter 3. The temporal profile of Tg-evoked Ca^{2+} signals is consistent with a slow, passive leak of Ca^{2+} from an intracellular pool. However, one or more rapid spikes could be seen superimposed on some slow-rising responses, and may represent events initiated by spontaneous release from Tg-sensitive stores (Missiaen et al., 1991). Consistent with a previous report on goldfish gonadotropes (Mollard and Kah, 1996), Tg also initiated Ca^{2+} signals in nominally Ca^{2+}-free media, but they were smaller and did not contain a sustained phase (average increase in $[Ca^{2+}]_c$ above baseline was ~25% of Tg-evoked Ca^{2+} signals in normal media; n=5), suggesting that a large component of the Tg-evoked Ca^{2+} signal is due to the subsequent influx of extracellular Ca^{2+}, presumably through SOC.

Ca^{2+} signals of maximally effective doses of sGnRH and cGnRH-II are known to result from the mobilization of intracellular Ca^{2+} stores (Johnson et al., 1999; Johnson et al., 2000; Chang et al., 2000). We have previously shown that 100 nM sGnRH or 100 nM cGnRH-II evoked Ca^{2+} signals with a rapid onset and rate of rise; a majority of responses were biphasic (containing distinct 'peak' and 'plateau' components; Johnson et al., 1999; Chapter 4). In order to evaluate Ca^{2+} signals initiated by directly activating intracellular Ca^{2+}-release channels, 10 mM caffeine was used. The temporal profile of caffeine-stimulated Ca^{2+} signals was variable and could not be classified as either biphasic or monophasic (Fig. 10.1C). Nonetheless, sGnRH, cGnRH-II (see Chapter 4) or caffeine all generated macroscopic Ca^{2+} signals with amplitude (~200% basal) comparable to those evoked by Tg (Fig. 10.1A,B). These data illustrate that goldfish gonadotropes can generate Ca^{2+} signals with very distinct rates of rise, but that the amplitude did not vary considerably between these treatments.

To control for cell-to-cell variability we confirmed these findings by applying Tg and either sGnRH, cGnRH-II or caffeine sequentially to the same cell and performing a paired analysis on the quantified kinetics of the Ca^{2+} signals (Fig. 1E). The results of these experiments are summarized in Table 10.1 and clearly show that the rate or rise of Tg-stimulated Ca^{2+} signals is significantly different from the other treatments in all cases. The whole cell Ca^{2+} signal latency and the mean increase in $[Ca^{2+}]_c$ were different in some cases, but these differences were not consistent and they depended on whether Tg was applied as the first or the second treatment. Collectively, these data indicated that the rate of rise is the major temporal difference between the Ca^{2+} signals of Tg, sGnRH, cGnRH-II, and caffeine.

Spatial comparison of Ca^{2+} signals

Spatial features of Ca^{2+} signals potentially carry functionally-important information. Thus, we examined the gross spatial distribution of the Ca^{2+} signals generated by the four treatments considered in this study. In the majority of cases (~85 %), Ca^{2+} signals evoked by Tg originated in the same cellular regions as those stimulated by either sGnRH or cGnRH-II (Fig. 10.1D). We confirmed this qualitative appraisal using a novel quantitative method that allows the comparison and pooling of spatial patterns within and between cells. On average, application of sGnRH, cGnRH-II and Tg all resulted in localized Ca^{2+} signals with similar quantified spatial parameters. The time-averaged gradient amplitude compared to basal for each treatment were as follows: sGnRH = 34 ± 9 nM, cGnRH-II = 39 ± 7 nM, Tg = 33 ± 10 nM; the direction/angle of the gradient relative to the nuclear region (set as 0^o) were as follows: sGnRH = 88 ± 10^o, cGnRH-II = 98 ± 14^o, Tg = 89 ± 13^o. Although sGnRH, cGnRH-II and Tg all generated Ca^{2+} signals with similar overall spatial characteristics, there were cell areas in which Ca^{2+} signals evoked by GnRH, but not Tg, propagated to. On the other hand, Ca^{2+} signals evoked by caffeine consistently arose from the region opposite the nucleus (data not shown), suggesting the mobilization of a Ca^{2+} pool that is spatially segregated from the major Tg- and GnRH-sensitive Ca^{2+} stores in gonadotropes. This represents a difference between Tg- and caffeine-evoked Ca^{2+} signals, other than the rate of rise. Therefore, we placed the greatest emphasis on comparisons between Tg and GnRH, intracellular Ca^{2+} store-mobilizing treatments which differ mainly in their temporal kinetics.

Comparison of GTH-II release

We examined the secretagogue activity of these treatments in separate cell-column perifusion experiments. We chose this approach to eliminate the confounding effects of Fura-2, a strong exogenous Ca^{2+} buffer, on the hormone release responses. Results from the present, as well as previous studies, have shown that Ca^{2+} signals generated by sGnRH and cGnRH-II differ with respect to their rate of rise (Johnson et al., 1999; Chapter 4). Keeping with the greater rate of rise of cGnRH-II-evoked Ca^{2+} signals, the size of cGnRH-II-stimulated GTH-II release responses was about twice that of those evoked by sGnRH (integrated area under the curve, sGnRH = 3358 ± 1143; cGnRH-II = 5950 ± 1380 cumulative % pretreatment; Fig. 10.2 A,B). As expected, directly activating Ca^{2+} release channels with 10 mM caffeine evoked robust GTH-II secretion (Fig. 10.2D). In contrast, treatment with Tg, which generated slow-rising Ca^{2+} signals, failed to evoke any GTH-II release. Surprisingly, Tg elicited a rapid and dramatic drop in GTH-II release (Fig. 10.2C). Collectively, these data show that passive Ca^{2+} signals evoked by the inhibition of SERCA with Tg evoke a strikingly different GTH-II release response when compared to the active release from intracellular Ca^{2+} triggered by the two GnRHs or caffeine.

Discussion

This is the first study to examine the importance of the rate of rise of Ca^{2+} signals in the control of hormone secretion evoked by multiple treatments which

act acutely by triggering the release of Ca^{2+} from intracellular stores. Slow-rising Ca^{2+} signals, generated by a Tg-revealed leak of Ca^{2+} from the ER, do not stimulate GTH-II exocytosis, whereas the rapid 'active' mobilization of Ca^{2+} stores by sGnRH, cGnRH-II or caffeine stimulates significant release. In goldfish, the acute actions of both GnRHs do not require the influx of extracellular Ca^{2+} through VGCC, but are dependent on intracellular Ca^{2+} stores (Johnson et al., 2000; Chapter 5). These Ca^{2+} stores are characterized by a novel pharmacology. Neither the Ca^{2+} signals (Mollard and Kah, 1996) nor the GTH-II release response (Chang et al., 2000; see Chapter 11) stimulated by GnRH is mediated by Tg-sensitive Ca^{2+} stores. Instead, GnRH-stimulated GTH-II release requires ryanodine-sensitive Ca^{2+} stores during most of the year (Chapter 8). The signal transduction of sGnRH, but not cGnRH-II, also involves additional Ca^{2+} stores which are sensitive to IP_3 and caffeine (Johnson et al., 2000; Chang et al., 1996; Chapter 5). Thus, we have compared treatments that rely on Ca^{2+} mobilization through at least three different signalling systems.

The physiological relevance of these findings is underscored by the observation that goldfish gonadotropes do not appear to use prominent frequency or amplitude coding of Ca^{2+} signals. Coordinated Ca^{2+} oscillations, that could be modulated in frequency, are not observed in this cell type (Johnson et al., 1999; Chapter 4). Several lines of evidence also indicate that Ca^{2+} signal maximal amplitude is not related to the effectiveness with which a given treatment stimulates GTH-II release. Although maximal doses of both GnRHs evoke Ca^{2+} signals of identical amplitude (this chapter; Johnson et al., 1999; Chapter 4), the acute GTH-II release is greater in response to cGnRH-II. Moreover, when compared to GnRH, PACAP which is a slightly weaker secretagogue, generates Ca^{2+} signals with greater amplitude (Chapter 4). Similarly, depolarization of gonadotropes with 30 mM KCl and subsequent activation of VGCC leads to Ca^{2+} signals that are approximately twice as large as GnRH (Johnson et al., 1999; Johnson et al., 2000), but evokes a smaller secretory response (Van Goor et al., 1998; JP Chang, unpublished observations).

Evidence from a variety of other cell types suggests that Ca^{2+} signal coding by the rate of rise may be more common and important than previously thought. Studies examining Ca^{2+} signals evoked by Tg- and hormones have generally found those elicited by Tg to have slower rates of rise (e.g. Ely et al, 1991). Although the Tg-induced Ca^{2+} signals, as well as their involvement in the signal transduction of agonists, have been examined to a considerable degree, actions of Tg on hormone release have received relatively little attention. In rat gonadotropes, secretion is positively coupled to Ca^{2+} released from Tg-sensitive stores and the rate of rise of Tg-evoked Ca^{2+} signals is dose-dependent (McArdle and Poch, 1992; Stojilkovic et al., 1993; Tse et al., 1997). Interestingly, Tg only stimulates GTH secretion at high doses (25 μM; McArdle and Poch, 1992) when rate of rise of its Ca^{2+} signals approximates those evoked by GnRH (Stojilkovic et al., 1993). Although Tg elicits exocytosis in other cell types (e.g. Cheek and Thastrup, 1989; Fomina and Nowycky, 1999), Tg does not stimulate basal secretion in long incubations in some model systems (e.g. atrial natriuretic peptide, Doubell and Thibault, 1994; α-subunit from αT3-1 cells, Holdstock et

al., 1996). However, $[Ca^{2+}]_c$ was not measured in the later systems. Perhaps the relative coupling of Tg-sensitive Ca^{2+} stores to secretion is dependent on the activation of SOC. Without the influence of store-dependent extracellular Ca^{2+} influx, Ca^{2+} signals generated from Tg-sensitive intracellular Ca^{2+} stores failed to induce exocytosis in mast cells (Kim et al, 1997) or chromaffin cells (Fomina and Nowycky, 1999). Interestingly, the activation of SOC has been correlated with the rate of Ca^{2+} mobilization from IP_3-sensitive Ca^{2+} stores (Machaca and Hartzell, 1999a). Our data suggest Tg evokes secondary extracellular Ca^{2+} entry in goldfish gonadotropes, as would be expected from all of the Ca^{2+} mobilizing treatments used herein.

In adrenal chromaffin cells, Cheek and co-workers (1989) demonstrated that secretagogue Ca^{2+} signals, generated as a result of nicotinic receptor activation, have distinct spatial patterns compared to those stimulated by IP_3-mobilizing muscarinic agonists (which are not secretagogues). However, examination of the published single-cell traces reveals that Ca^{2+} signals evoked by nicotinic receptor-induced VGCC activity are initiated faster and have a more rapid rate of rise than Ca^{2+} signals that do not lead to secretion. Similarly, it has been suggested that the rate of rise was an important factor in the regulation of prolactin secretion from GH_4C_1 cells responding to depolarization or hyposmotic shock (Sato et al., 1990). In addition, although Tg and thyrotropin-releasing hormone generated Ca^{2+} signals of similar amplitude in rat anterior pituitary cells, Tg was only weakly coupled to prolactin and thyroid-stimulating hormone secretion (Sato et al., 1992). In the latter study, the rate of rise of Ca^{2+} signals generated by Tg, though it was not quantified, also seemed to be slower than those evoked by TRH.

Why do slow-rising Ca^{2+} signals fail to stimulated secretion, in spite of attaining similar amplitudes as secretagogue treatments? It is possible that slower increases in $[Ca^{2+}]_c$, such as those generated by Tg in the present study, may be insufficient to activate Ca^{2+}-induced Ca^{2+} release (CICR; Hernandez-Cruz et al., 1997), thereby failing to transmit the signal to microdomains containing the Ca^{2+} sensor for secretion. Due to the very low effective diffusion coefficient of Ca^{2+} in cytosol, the action of Ca^{2+} is likely to be local in goldfish gonadotropes, as in other cell types (Allbritton et al., 1992; Clapham, 1995). Furthermore, the cytoplasm of goldfish gonadotropes is packed with ER stacks, secretory granules, large globules and other organelles that would serve as barriers to the passive diffusion of Ca^{2+} (Chang et al., 1990). The prominence of Ca^{2+} gradients in 'resting' and stimulated goldfish gonadotropes (this Chapter, Chapter 3) suggests that Ca^{2+} wave propagation is very tightly controlled in this cell type. Thus, data presented herein support a scenario where Ca^{2+} signal propagation through CICR (and/or other means) would only be active in certain specialized situations.

How are increases in $[Ca^{2+}]_c$ coupled to decreases in hormone release? Potential answers are considered in Chapter 11. Interestingly, dopamine, a well characterized inhibitor of GTH-II release (Van Goor et al., 1998; Chang et al., 2000) also generated very slow-rising Ca^{2+} signals (rate: 0.15 ± 0.1 nM/sec). These were not directly compared to the other Ca^{2+} mobilizing treatments in the present study because the increases in $[Ca^{2+}]_c$ were relatively small (amplitude:

40 ± 17.2 nM above baseline; n=5) and because we have no information on their mechanism. Collectively, these findings suggest that Tg-sensitive Ca^{2+} stores may not be positively coupled to hormone secretion in some cell types. The fact that Tg generally evokes slow-rising Ca^{2+} signals provides a possible explanation for this paradox. To our knowledge, the only precedent in the literature for the ability of Tg to decrease hormone secretion is the case of parathyroid hormone release, which is thought to be negatively coupled to increased Ca^{2+} *per se* (Shoback et al., 1995).

Previous work on temporal coding of Ca^{2+} signals has primarily focused on cell types exhibiting regular $[Ca^{2+}]_c$ oscillations. However, virtually nothing is known about how Ca^{2+} signals in non-oscillating systems impart functional specificity. Findings contained in this chapter suggest that the relative degree of GTH-II secretion between two closely-related neuropeptides may be related to the rate of rise, and not the amplitude, of their Ca^{2+} signals. Furthermore, an extreme case is presented, where the very slow-rising Ca^{2+} signals generated by Tg are apparently insufficient to evoke exocytosis, but instead lead to a paradoxical decrease in GTH-II release. This report presents novel evidence that the rate of rise can encode important information within Ca^{2+} signals.

TABLE 10.1. Quantitative comparison of secretagogue- and Tg-generated Ca^{2+} signals in gonadotropes. Quantitative parameters are calculated as described in the Chapter 3. cGnRH-II (100 nM), sGnRH (100 nM), Caff (10 mM), or Tg (2 μM) were applied sequentially, as indicated, to identified gonadotropes in 5-minute pulses separated by a 6-minute recovery. All values are presented as mean ± SEM.

Treatment‡	Kinetic Parameter			
	Latency (s)	Rate of Rise (nM/s)	Max. Ampl. (nM)	Mean Ca^{2+} (nM)
cGnRH-II	22 ± 3	4.9 ± 1.9	177 ± 50	95 ± 20
Tg	49 ± 6*	0.9 ± 0.2*	103 ± 13	161 ± 25*
Tg	62 ± 24	0.9 ± 0.3*	161 ± 63	80 ± 90
cGnRH-II	39 ± 14	1.7 ± 0.9	145 ± 30	77 ± 12
sGnRH	18 ± 3	1.5 ± 0.3	161 ± 15	96 ± 10
Tg	69 ± 24*	0.8 ± 0.3*	96 ± 15	50 ± 8*
Tg	46 ± 11	1.4 ± 0.3*	151 ± 27	75 ± 16
sGnRH	40 ± 8	2.2 ± 0.4	209 ± 41	107 ± 21
Tg	72 ± 26	1.1 ± 0.3*	252 ± 41	98 ± 17*
Caff	21 ± 4	9.3 ± 5.8	253 ± 42	194 ± 68

‡ the number of replicates for each experiment are as follows: n = 15 cGnRH-II vs Tg; n = 7 Tg vs cGnRH-II; n = 13 sGnRH vs Tg; n = 11 Tg vs sGnRH; n = 5 Tg vs Caff

* denotes significant difference compared to paired secretagogue stimulus using Wilcoxon signed-rank test

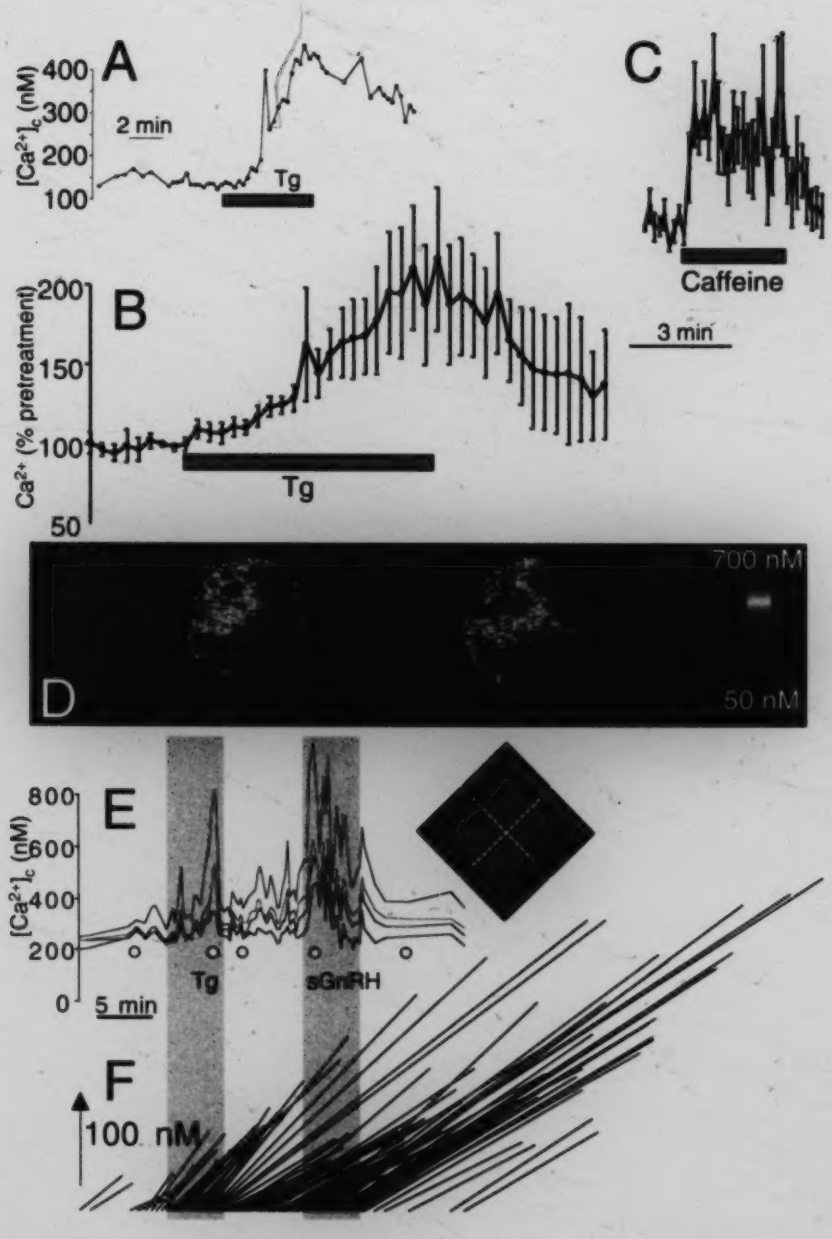
A
400
300
200
100
$[Ca^{2+}]_c$ (nM)
2 min
Tg
C
Caffeine
3 min
B
200
150
100
50
Ca^{2+} (% pretreatment)
Tg
700 nM
D
50 nM
E
800
600
400
200
0
$[Ca^{2+}]_c$ (nM)
Tg
sGnRH
5 min
F
100 nM

Figure 10.1. Analysis of temporal profiles and spatial patterns of Ca^{2+} signals evoked from mobilization of intracellular Ca^{2+} stores in goldfish gonadotropes. A) Representative trace of $[Ca^{2+}]_c$ increase generated by 2 μM Tg. B) Pooled Tg-stimulated Ca^{2+} signals (n=10). C) Pooled Ca^{2+} signals elicited by 10 mM caffeine (n=6). Time and amplitude scales for C-E are as in B. Treatments were applied as indicated by horizontal blue bars. D) False-colour images of Ca^{2+} signals evoked by Tg or sGnRH, applied sequentially to the same gonadotrope. Time of images is indicated in G (*yellow dots*). E) Representation of the $[Ca^{2+}]_i$ estimated for four quadrants (for colour code, see inset). Tg and GnRH are applied sequentially, as indicated (*gray areas*). F) Vector representation of the Tg- and GnRH-stimulated Ca^{2+} signals. Scale arrow indicates a 100 nM gradients emanating from the nuclear end of the cell. *Inset* The observable morphological characteristics and colour code for quadrants are shown for the gonadotrope analyzed in E-F.

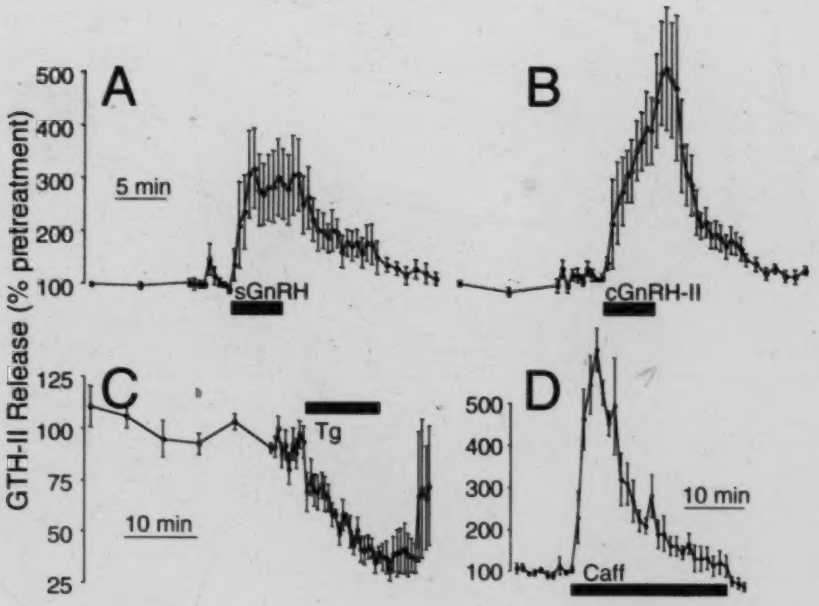

Figure 10.2. Comparison of GTH-II release response to treatments (*black bars*) generating Ca^{2+} signals with different rate kinetics. Cells were treated with 100 nM sGnRH (A, n=6), 100 nM cGnRH-II (B, n=10), 2 μM Tg (C, n=6), 10 mM caffeine (D, n=6) in similar, but independent experiments.

Chapter 11.

Function-specific intracellular Ca^{2+} stores control multiple components of the extended gonadotropin secretory pathway

From gene transcription to exocytosis, Ca^{2+} plays a role in multiple components of the extended secretory pathway (see Chapter 1). The contribution of cytosolic Ca^{2+} levels ($[Ca^{2+}]_c$) to the control of the secretory system has been well established, especially in the case of evoked exocytosis (Neher, 1998). $[Ca^{2+}]_c$ has also been implicated in the recruitment of secretory vesicles/granules into the releasable pool (Smith et al., 1998), as well as constitutive membrane fusion (Peters and Mayer, 1998). In addition, there is growing appreciation that the lumenal Ca^{2+} environment ($[Ca^{2+}]_L$) of the organelles along the secretory pathway (ER, Golgi, secretory granules) may regulate the production and release of hormones at multiple sites (reviewed in Johnson and Chang, 2000a; Chapter 1). For example, the $[Ca^{2+}]_L$ of secretory vesicles/granules may modulate the condensation state of peptide hormones and the probability of exocytosis (Han et al., 1999; Scheenen et al., 1998) in neuroendocrine cells.

Endoplasmic reticulum (ER) Ca^{2+} stores also have multiple functions in the secretory pathway. On one hand, cytosolic Ca^{2+} signals from the ER trigger exocytosis in many cell types (Petersen et al., 1994; Pozzan et al., 1994; Tse et al., 1997). On the other hand, lumenal Ca^{2+} homeostasis must be sustained for the maintenance of normal post-translational processing, folding, sorting and packaging of secreted proteins (Austin and Shields, 1996; Canaff et al., 1996; Di Jeso et al., 1997). Cells in which ER $[Ca^{2+}]_L$ has been depleted with Tg, exhibit decreased levels of certain proteins within the ER. This phenomenon may be due to the stimulation of proteolysis and/or increased transport out of the ER (Wileman et al., 1991; Jeffery et al., 2000). Tg treatment has also been shown to block the export of certain misfolded proteins from the ER (Di Jeso et al., 1997). ER $[Ca^{2+}]_L$ may also regulate mRNA translation. Depletion of ER Ca^{2+}, with Tg or hormone stimulation, leads to an inhibition of translation initiation within minutes (Brostrom and Brostrom, 1990; Reilly et al., 1998). Interestingly, protein synthesis in HeLa cells was only sensitive to specific Ca^{2+}-mobilizing treatments (Preston and Berlin, 1992), suggesting a functional heterogeneity between multiple ER Ca^{2+} stores.

Ca^{2+} regulates protein production at the level of gene transcription through a large array of positive and negative Ca^{2+}-dependent transcription regulators (Chapter 1). Based on the known literature, the inhibition of gene expression seems to be less common than the Ca^{2+}-dependent stimulation of gene expression. In a scenario reminiscent of the control of translation, perturbed $[Ca^{2+}]_L$ homeostasis alters the expression of genes encoding ER resident Ca^{2+} buffering proteins and chaperones, such as calreticulin and BiP, via a signal which does not require $[Ca^{2+}]_c$ elevation (Llewellyn et al., 1996). Clearly, the regulation of multiple components of the extended secretory pathway by Ca^{2+} is complex. How does Ca^{2+} regulate all of these cellular functions with specificity?

We have proposed that cells utilize multiple, function-specific Ca^{2+} stores to regulate distinct components of the extended secretory pathway (see Chapter

1). A comprehensive review of the existing literature indicates that most cells are likely to possess multiple intracellular Ca^{2+} stores (reviewed in Johnson and Chang, 2000a; Chapter 1). Moreover, intracellular Ca^{2+} pools can be subdivided into several pharmacological classes, including one mobilized by IP_3 and refilled by SERCA pumps (the major agonist-sensitive Ca^{2+} store in most cell types; Pozzan et al., 1994; Petersen et al., 1994). A second major class of ER Ca^{2+} store is sensitive to Ry (Ehrlich et al., 1994; Sorrentino and Volpe, 1993). Although the involvement of Tg-sensitive Ca^{2+} stores in several components of the secretory pathway has been studied, less is known about the role of Ry-sensitive stores in these processes. Specifically, the role of Ry-sensitive Ca^{2+} stores in the control of gene expression has not received considerable attention in non-muscle cells.

Functional studies of basal and agonist-evoked hormone secretion have indicated that goldfish gonadotropes may possess multiple classes of intracellular Ca^{2+} stores (Johnson et al., 2000; Chapters 5,8). Surprisingly, GnRH-stimulated GTH-II release required intracellular Ca^{2+} pools sensitive to Ry (Chapter 8), rather than the classical IP_3- and Tg-sensitive Ca^{2+} stores which mediate agonist-signalling in many cell types, including mammalian gonadotropes (Tse et al., 1997). Indeed, Mollard and Kah (1996) have shown that GnRH-evoked Ca^{2+} signals were not blocked by Tg in goldfish gonadotropes, although the presence of Tg-sensitive Ca^{2+} pools was confirmed. Together, these findings suggest that Ry- and Tg-sensitive Ca^{2+} stores co-exist in goldfish gonadotropes, but are differentially involved in agonist-signalling.

In this study, a combination of experimental approaches was used to demonstrate the differential involvement of Tg-sensitive Ca^{2+} stores and Ry-sensitive Ca^{2+} stores in the regulation of various aspects of the secretory pathway, including 1) acute GnRH-stimulated GTH-II release, 2) acute basal GTH-II release, 3) long-term GTH-II secretion, 4) long-term GTH-II storage and 5) GTH-II gene expression.

Results

Tg-sensitive Ca^{2+} stores are not involved in GnRH-stimulated GTH-II release

Although a previous report has indicated that GnRH-evoked Ca^{2+} signals are Tg-insensitive (Mollard and Kah, 1996), the effects of Tg on basal and GnRH-stimulated hormone release have not been studied. In the present study, the role of Tg-sensitive Ca^{2+} stores in GnRH-stimulated GTH-II release and other gonadotrope functions was examined. First, we confirmed the presence of Tg-sensitive Ca^{2+} stores in individual gonadotropes. Indeed, 2 μM Tg (a dose which inhibits all known SERCA isoforms; Lytton et al., 1991) evoked slow-rising Ca^{2+} signals, consistent with a passive leak from intracellular Ca^{2+} stores revealed upon the inhibition of SERCA (Fig. 11.1). Compared to Ca^{2+} signals generated by GnRH (Chapter 4; Johnson et al., 1999), Tg-evoked Ca^{2+} signals were of comparable amplitude (252 ± 41 nM above baseline), but invariably had a slower rate of rise (see Chapter 10). In most cells, $[Ca^{2+}]_c$ was maintained at a noticeably higher level after the removal of Tg. This long-term effect often persisted for the duration of our recording (usually ~ 30 minutes).

Next, we monitored the effects of 2 μM Tg on GTH-II release responses evoked by a maximally effective dose of sGnRH or cGnRH-II (100 nM; Chang et al., 1990). Compared to both sequential and parallel controls, Tg had no effect on GTH-II release responses stimulated by either sGnRH or cGnRH-II (Fig. 11.2). Likewise, 2 μM Tg did not affect the GTH-II release evoked by 10 mM caffeine, which is thought to mobilize intracellular Ca^{2+} directly from sGnRH-sensitive Ca^{2+} store(s) in goldfish gonadotropes (Johnson et al., 2000; Chapter 5, 6). Collectively, these data demonstrate that although Tg-sensitive Ca^{2+} stores are present in goldfish gonadotropes, they are not involved in acute GnRH- or caffeine-stimulated GTH-II release.

Tg-sensitive Ca^{2+} stores mediate GTH-II release from unstimulated cells

Next, the role of Tg-sensitive Ca^{2+} stores in the control of basal GTH-II release was investigated. In spite of the observations that 2 μM Tg elevates $[Ca^{2+}]_c$ (Fig. 11.1), a robust, rapid and reversible decrease in basal GTH-II release was observed after application of Tg; this was followed by a significant 'rebound' response (Fig. 11.3A). A similar pattern of hormone release was measured when samples were collected every 30 seconds (Chapter 10), eliminating the possibility that an early substantial increase in GTH-II secretion occurred and was missed in the 5-minute fraction collection protocol. Similarly, several other doses of Tg also reduced basal GTH-II release in perifusion experiments (60-second sample collection rate; Fig. 11.3B). These results clearly indicate that global $[Ca^{2+}]_c$ elevations induced by Tg is not correlated with an increase in GTH-II exocytosis. Interestingly, the pronounced decrease in GTH-II release induced by Tg was not observed in cell columns which had been transiently pre-exposed to sGnRH. A 5-minute challenge with sGnRH (Fig. 11.2A, 11.3C), but not cGnRH-II (Fig. 11.3C), rescued the inhibition of basal hormone release induced by Tg applied 55 minutes later. Similarly, caffeine-treatment abrogated the GTH-II response to Tg (Fig. 3D). These observations suggest the presence of a one-way interaction between sGnRH signalling and Tg-sensitive Ca^{2+} stores, despite the non-involvement of Tg in GnRH-stimulated GTH-II release.

Since protein kinases cascades are important components of long-term GnRH signalling in goldfish gonadotropes (Chang et al., 1996; Chapter 3), we speculated based on these interactions, that Tg-evoked Ca^{2+} signals might have inhibited basal GTH-II secretion by altering the phosphorylation state of proteins important in exocytosis through the activation of a phosphatase. However, okadaic acid (0.5 μM), a potent and specific Ser/Thr phosphatase inhibitor (Dawson and Holmes, 1999), had no effect on the GTH-II release response elicited by Tg (Fig. 11.3E), suggesting that activation of these phosphatases does not underlie the actions of Tg on basal GTH-II release.

Characterization of long-term GTH-II release and storage

In preparation for experiments examining the role(s) of Tg-sensitive Ca^{2+} stores and Ry-sensitive Ca^{2+} stores on upstream components of the extended secretory pathway, a characterization of long-term GTH-II secretion and storage in unstimulated cultures was performed. Mixed cultures of goldfish pituitary cells

were incubated in testing media for 2, 12 or 24 hours, after which media was collected and the cells were lysed. Over this time period, gonadotropes store relatively little immunoreactive GTH-II compared to the amount secreted (Fig. 11.4A). Over the course of 24 hours, the amount of GTH-II contained within the cells remained relatively stable (a decrease in apparent production was sometimes seen at the 12 hour time point), but the rate of GTH-II release dropped significantly (2 hours, 2349 ± 198 ng/hour; 12 hours, 571 ± 41 ng/hour; 24 hours, 257 ± 58 ng/hour). Although the absolute amount of GTH-II released and stored varied between different experiments, the pattern of release and the relative amount of stored hormone did not. Therefore, to facilitate the pooling of results from separate experiments, GTH-II values were normalized to their time-matched control cultures (% control) in subsequent analysis. The basal characteristics of GTH-II release and storage were not affected by the drug vehicles used in the present study (Fig. 11.4B). In contrast, 100 µM cycloheximide reduced GTH-II secretion and total GTH-II production (secretion + contents) at 12 and 24 hours. These data imply that gonadotropes begin to use newly synthesized GTH-II by 12 hours.

Some cells release newly synthesized hormones through the constitutive secretory pathway, where small vesicles of newly produced proteins bud off from the *trans* Golgi network and proceed directly to the plasma membrane. These vesicles are not stored like granules of the regulated secretory pathway, but instead, immediately fuse and undergo exocytosis in the absence of a measurable global Ca^{2+} signal (Kelly et al., 1985). Farnworth (1995) has hypothesized that mammalian gonadotropes may release a portion of their hormone through the constitutive secretory pathway. This pathway usually results in the release of proteins within ~1 hour of synthesis (Moore, 1987). To examine whether basal GTH-II release occurs via this mode of secretion, we tested the effects of cycloheximide, a well-characterized inhibitor of protein synthesis. Prolonged exposure to 100 µM cycloheximide did not reduce GTH-II release in 2-hour static incubation studies (Fig. 11.4B). This was further confirmed in time-resolved perifusion experiments where long treatments of cycloheximide had no effect on either basal or evoked GTH-II release (Fig. 11.4C). Furthermore, neither basal nor GnRH-stimulated GTH-II release were affected by 10 µM brefeldin A (Fig. 11.4D), which blocks constitutive secretion by selectively disrupting the Golgi apparatus (Chardin and McCormick, 1999). These results argue against a prominent role for the constitutive secretory pathway in the release of GTH-II during the first 2 hours. Similarly, brefeldin A did not alter Tg-induced decreases in basal GTH-II release (Fig. 11.4E).

Differential modulation of long-term GTH-II release and storage by distinct intracellular Ca^{2+} stores

Next, I compared the long-term effects of perturbing different Ca^{2+} stores using Tg, as well as two doses of Ry which are known to have opposite effects in RyR function (Ehrlich et al., 1994; Chapter 8). Overall, the major effect of 2 µM Tg treatment was a reduction in GTH-II contents (Fig. 11.5). The reduced cellular GTH-II levels may have been related to the increased secretion seen over the first

12 hours, rather than a reduction of total GTH-II production. On the other hand, activation of RyR with 1 nM Ry resulted in robust GTH-II release over 2 hours, with a concomitant increase in acute GTH-II production. After a transient accumulation of cellular GTH-II (seen at 12 hours), the stimulatory Ry treatment resulted in a pronounced decrease in GTH-II release, storage and total production by 24 hours (Fig. 11.5). In contrast, inhibition of RyR with 10 μM Ry transiently suppressed GTH-II release and production during the first 12 hours, but not over 24 hours (Fig. 11.5). Examination of the data at each timepoint revealed clear differences in the roles of Tg-sensitive Ca^{2+} stores and Ry-sensitive Ca^{2+} stores in the extended secretory pathway. After 2 hours, only 1 nM Ry had a stimulatory effect on gonadotropes, while Tg-treated cells showed a tendency towards a reduction in GTH-II contents and release. However, by 12 hours, depletion of Tg-sensitive Ca^{2+} pools had resulted in the apparent secretion of stored GTH-II, whereas mobilization of Ry-sensitive Ca^{2+} stores had induced an accumulation of cellular GTH-II, without further activation of secretion. At the same time, inhibition of RyR led to a marked decrease in GTH-II release and production. Over 24 hours, Tg continued to reduce stored GTH-II, by a mechanism that did not involve a significant reduction in release or total production. Similarly, GTH-II contents were significantly reduced (60% of control) over 24 hours in cultures treated with 20 μM Tg (data not shown). Although the stimulatory dose of Ry also reduced stored GTH-II over 24 hours, this was accompanied by significant decreases in GTH-II release and net production. Interestingly, the long-term result of blocking Ca^{2+} release from Ry-sensitive ER is a net accumulation of cellular GTH-II. Taken together, these results strongly suggest that specific aspects of the extended secretory pathway can be regulated independently by different Ca^{2+} stores.

Differential modulation of GTH-II β subunit mRNA expression by distinct intracellular Ca^{2+} stores

GTH-II is a glycoprotein dimer. The α-subunit is shared with GTH-I (and thyrotropin), and is thought be present in excess, whereas the β subunit is hormone-specific (Yoshiura et al., 1997). As was the case with GTH-II protein levels, perturbing Tg- or Ry-sensitive Ca^{2+} stores also modulated GTH-II β subunit mRNA levels. After a delay, 2 μM Tg induced a reduction in GTH-II gene expression by 12 and 24 hours, relative to time-matched controls (Fig. 11.6). The effects of both stimulatory and inhibitory doses of Ry were biphasic, eventually leading to a decrease in GTH-II mRNA. Clear differences in the functional roles of multiple Ca^{2+} stores were found by comparing the effects of all three treatments on mRNA accumulation at each timepoint. After 2 hours, neither Tg nor 1 nM Ry, treatments that would be expected to results in enhanced release of Ca^{2+} from stores, induced a major change in GTH-II mRNA from its steady state levels. In contrast, blocking RyR-mediated Ca^{2+} mobilization with 10 μM Ry significantly increased GTH-II β subunit mRNA expression. Over 12 hours, release of Ca^{2+} from Tg-sensitive Ca^{2+} stores caused a decrease in gene expression, whereas Ca^{2+} release from RyR caused an increase. Over the entire 24 hours, the net effect of all the treatments was to induce a decrease in GTH-II β-

subunit accumulation. Nonetheless, the net effects of Tg and 1 nM Ry were significantly different from one another over the duration of the experiment ($P < 0.05$).

Negative coupling of $[Ca^{2+}]_c$ to GTH-II gene expression

Differences between the effects of 1 nM Ry and 10 μM Ry, as well as between Tg and 1 nM Ry, on GTH-II β subunit levels suggest that changes in $[Ca^{2+}]_c$ and $[Ca^{2+}]_L$ may be differentially coupled to GTH-II gene expression. To facilitate the understanding of such interactions, we examined the role of $[Ca^{2+}]_c$ signals, generated independently of intracellular Ca^{2+} stores (Jobin and Chang, 1992), on GTH-II β-subunit mRNA expression. Cells were challenged with 30 mM KCl for 30 min to activate VGCCs; GTH-II mRNA was measured after 12 hours. We chose this short treatment protocol because chronic exposure to high KCl (6-24 hours) causes the detachment of cells in our cultures (JP Chang, unpublished observations). GTH-II β subunit mRNA levels were dramatically reduced following activation of Ca^{2+} entry through VGCC (Fig. 11.7). This result suggests that elevated $[Ca^{2+}]_c$ initiates a signal that is inhibitory to GTH-II gene expression.

Modulation of long-term GTH-II release and storage by GnRH, cAMP and other SERCA inhibitors

Next, the long-term effects of other known modulators of acute GTH-II release were investigated. Treatment of cultures with 100 nM sGnRH or 100 nM cGnRH-II for 24 hours significantly stimulated GTH-II release. Unlike long-term exposure to Ca^{2+} mobilizing treatments, such as Tg or 1 nM Ry (Fig. 11.5), GnRH did not induce a decrease in cellular GTH-II contents (Fig. 11.8A). Although not involved in GnRH-signalling, the cAMP/protein kinase A (PKA) pathway mediates the GTH-II-releasing actions of PACAP (Chang et al., 2000; JP Chang, NR Wirachowsky, P Kwong, JD Johnson, unpublished). Forskolin (10 μM), which activates an early step (adenylate cyclase) in this signalling system, increased the release and net production of GTH-II over 24 hours (Fig. 11.8B). In addition, there was a slight, but significant decrease in cellular GTH-II storage. Collectively, these data show that certain agonist signalling pathways can be coupled to prolonged increases in GTH-II release, without necessarily reducing cellular GTH-II contents.

BHQ and CPA are structurally-unrelated SERCA inhibitors that are thought to act on intracellular Ca^{2+} stores identical to those affected by Tg (although Tg is significantly more potent; Mason et al., 1991). The finding that GnRH-stimulated GTH-II release was Tg-insensitive (this Chapter) illuminated a potential contradiction in our model since we have previously reported that GnRH signalling was partially inhibited by BHQ (sGnRH- and cGnRH-II-evoked GTH-II release reduced to 32% and 33% of controls, respectively) and CPA (sGnRH- and cGnRH-II-evoked GTH-II release reduced to 32 % and 8 % of control, respectively; Johnson et al., 2000; Chapter 5). In the present study, we investigated whether the long-term effects of Tg, BHQ and CPA were also divergent. Unlike 2 μM Tg, treatment with 10 μM BHQ or 10 μM CPA

stimulated GTH-II release over 24 hours (Fig. 11.9). Tg and BHQ, but not CPA, significantly reduced GTH-II storage. Only CPA treatment resulted in a significant increase in net GTH-II production. These data suggest, by virtue of their different functional consequences, that these three inhibitors may have distinct molecular targets, possibly different SERCA isoforms.

Discussion

Goldfish gonadotropes possess multiple intracellular Ca^{2+} stores

Results from this chapter provide novel evidence to support the hypothesis that distinct intracellular Ca^{2+} stores can be differentially coupled to specific Ca^{2+}-dependent components of the extended secretory pathway in neuroendocrine cells. From this hypothesis, one would predict that certain Ca^{2+} stores may be functionally uncoupled from specific Ca^{2+}-dependent events. Accordingly, although Tg-sensitive Ca^{2+} stores are present in goldfish gonadotropes, these Ca^{2+} stores are not involved in acute GTH-II release evoked by two endogenous agonists, sGnRH and cGnRH-II. Although GnRH signalling in goldfish somatotropes is also Tg-insensitive (Johnson and Chang, 2000b; Chapter 7), these findings were nonetheless unexpected because most intracellular Ca^{2+}-mobilizing agonists studied thus far employ a prototypical IP_3- and Tg-sensitive Ca^{2+} pool (Petersen et al., 1994; Pozzan et al., 1994; Tse et al., 1997). For example, in rat gonadotropes, mGnRH-stimulated GTH release is abolished by 1 μM Tg; only high concentrations of Tg (25 μM) evoke GTH release (McArdle and Poch, 1992). In contrast, both GnRHs require intact Ry-sensitive Ca^{2+} stores to control goldfish GTH-II release, except during sexual maturation when sGnRH-stimulated GTH-II release becomes Ry-insensitive (Chapter 8). In the present study, GnRH-stimulated GTH-II release was Tg-insensitive throughout the yearly reproductive cycle. These results, taken together with the findings that BHQ and CPA modulated GTH-II release and total GTH-II production in manners distinct from Tg, clearly indicate the presence of multiple intracellular Ca^{2+} stores capable of differentially modulating events in the secretory pathway in goldfish gonadotropes. Whether BHQ and CPA-sensitive Ca^{2+} stores correspond to the Ry-sensitive Ca^{2+} stores that mediate acute GnRH signalling remains to be determined. The observed and deduced functional properties of intracellular Ca^{2+} stores in goldfish gonadotropes (as further discussed below) differ substantially from those reported for mammalian gonadotropes (Stojilkovic and Catt, 1995).

Tg-sensitive Ca^{2+} stores control basal secretion

The inability of Tg to acutely stimulate basal GTH-II release provides further evidence that some Ca^{2+} pools are not positively coupled to late events along the secretory pathway. The uncoupling of Ca^{2+} signalling from secretion has also been suggested for αT3-1 cells (Trueta et al., 1999). Surprisingly, Tg treatment actually led to a rapid and reversible decrease in basal GTH-II release in our model system. Other than parathyroid cells, where elevated $[Ca^{2+}]_c$ *per se* is thought to be inhibitory to hormone release (Shoback et al., 1995), this the first demonstration of acute Tg-induced inhibition of basal hormone release. Although α-subunit release from αT3-1 cells was reduced by exposure to 1 μM Tg (assayed

after 24 hours; Holdstock et al., 1996), it is not clear whether this reflects an acute response. In the present study, the acute inhibitory GTH-II release response may be specific to Tg-sensitive Ca^{2+} stores, since acute Ry treatment (10 μM) did not mimic the inhibitory effects of Tg on basal secretion (Chapter 8). Moreover, growth hormone release, measured under the similar conditions, is not substantially affected by Tg treatment (Johnson and Chang, 2000b; Chapter 12), suggesting a degree of cell specificity. To our knowledge, this is the first demonstration of an intracellular Ca^{2+} store that specifically blocks basal hormone release when perturbed, without affecting agonist-evoked exocytosis.

I have made many attempts to discern the mechanism by which acute Tg treatment inhibits basal GTH-II release. Several possibilities have been considered, including that 1) Tg-evoked Ca^{2+} signals actually encode an inhibitory signal (possibly by modulating phosphorylation cascades), 2) Tg may inhibit a component of the constitutive secretory pathway, and 3) Tg may inhibit the spontaneous fusion of secretory granules by reducing their lumenal $[Ca^{2+}]$. The first possibility is difficult to reconcile with the observation that elevations in $[Ca^{2+}]_c$ stimulated by Tg have roughly the same amplitude as Ca^{2+} signals generated by sGnRH or cGnRH-II (Johnson et al., 1999). Notwithstanding, investigations into the spatial and temporal features of Ca^{2+} signals that may correlate with the control of exocytosis are ongoing (Chapter 10). Although our data indicate that okadaic acid-sensitive phosphorylation cascades do not mediate the Tg-induced decrease in GTH-II release, the possibility that okadaic acid-insensitive phosphatases are involved also needs to be addressed in future studies.

The second possibility also seems unlikely given the present findings. Although mutations in *PMR1*, a Ca^{2+}ATPase, blocks protein transit through the secretory pathway in yeast (Rudolph et al., 1989), Tg reduced GTH-II release after only 30 seconds. This observation rules out the possibility that the Tg response is caused by the blocking of early events in the extended secretory pathway (gene expression, protein synthesis, post-translational modifications or sorting) that may modulate secretion if GTH-II was released through a constitutive secretory pathway. Moreover, results from experiments with cycloheximide and brefeldin A strongly suggest that basal (or evoked) GTH-II release does not follow the constitutive secretory pathway during this timeframe. Thus, it is likely that basal GTH-II release occurs exclusively as a result of a random fusion of GTH-II-containing secretory granules in the regulated secretory pathway. As is the case with this form of basal secretion in mammalian pituitary cells (Varro et al., 1996), basal GTH-II release is temperature-sensitive in goldfish (Chang and Jobin, 1994; JP Chang, unpublished observations).

On the other hand, the third possibility seems more likely. The timecourse and insensitivity to brefeldin A of Tg-induced decrease in basal GTH-II release suggests a post-Golgi mechanism. Perhaps Tg modulates $[Ca^{2+}]_L$ in a population of secretory granules. Whether secretory granules are physiologically-relevant Ca^{2+} stores and contain Tg-sensitive SERCA are controversial questions, with answers that probably depend on the cell type (Nicaise et al., 1992; Martinez et al., 1996; Gerasimenko et al., 1996a; Blondel et al., 1996; Jonas et al., 1997; Nguyen et al., 1998). Recently, Peters and Mayer (1998) demonstrated that the

local release of lumenal Ca^{2+} controls a calmodulin-mediated late event in yeast vacuole fusion, a constitutive fusion process with many similarities to exocytosis. Like basal hormone release, this process does not require a large global Ca^{2+} signal. Importantly, Tg treatment renders vacuoles fusion-incompetent (Peters and Mayer, 1998). Therefore, the depletion of secretory granule $[Ca^{2+}]_L$ is a possible post-Golgi mechanism of action for Tg in goldfish gonadotropes. It is important to note that EM studies have shown that goldfish gonadotropes possess at least two morphologically-distinct populations of secretory granules, as well as large globules (Nagahama, 1973; Kaul and Vollrath, 1974; discussed in Chapter 6). Whether any of these granule populations are selectively involved in basal GTH-II release, agonist-evoked GTH-II release or Ca^{2+} signalling, remains to be determined.

At this point, however, one can only conclude that the acute Tg-induced inhibition of basal GTH-II release involves signalling events that are sensitive to pre-exposure to sGnRH, but not cGnRH-II. It is tempting to speculate that this results from one of the known differences in signal transduction cascades activated by the two GnRHs, such as the participation of caffeine-sensitive, depletion-resistant Ca^{2+} stores in sGnRH-stimulated GTH-II release (Chang et al., 1995; Chang et al., 1996; Johnson et al., 1999; Johnson et al., 2000). The observation that caffeine also prevents the Tg-induced decreases in basal GTH-II release suggests an important role for this agonist-specific Ca^{2+} store. The apparent features of this caffeine-sensitive Ca^{2+} store are consistent with the possibility that they are closely associated with or within secretory granules (see Chapters 5,6). Collectively, these data form the basis for the hypothesis that a secretory granule-associated Ca^{2+} store, which can be modulated in some way by sGnRH and caffeine, may interact with the mechanisms mediating the Tg-induced decrease in GTH-II release. However, clear evidence of Tg-mediated decreases in secretory granule $[Ca^{2+}]_L$, and the modulation of their Ca^{2+} loading state by prior exposure to sGnRH or caffeine, is required to test this hypothesis.

Differential control of long-term GTH-II release, storage and production

In the present study, we also compared the roles of multiple intracellular Ca^{2+} stores on the long-term release, storage and production of immunoreactive GTH-II. Depletion of Ca^{2+} in Tg-sensitive stores caused the emptying of stored GTH-II without a compensatory increase in production. However, unlike cycloheximide, which also reduced GTH-II cell contents, Tg did not produce a concomitant decrease in apparent GTH-II production or a long-term suppression of secretion. Thus, it is likely that perturbing/depleting Tg-sensitive ER Ca^{2+} pools accelerates the degradation of GTH-II and/or the export of GTH-II into a releasable pool of secretory granules, as has been proposed for other cell types (Wileman et al., 1991; Jeffery et al., 2000). That Tg transiently elevated GTH-II release at 12 hours is consistent with this second alternative. With this interpretation, the apparent secretion evoked by Tg over 12 hours does not directly contradict the acute effect of Tg, which is the reduction of basal secretion. Given the possibility that Ca^{2+} may be an important regulator of multiple processes at multiple sites along the extended secretory pathway, this hypothesis

does not seem unlikely. Although activation of RyR over 24 hours also reduced GTH-II contents, two lines of evidence suggest that Ry does not act through the same mechanisms as Tg. First, unlike Tg, 1 nM Ry also significantly reduced long-term GTH-II release, as well as production. Second, Tg- and Ry-induced changes in cellular GTH-II content display markedly different kinetics, as the inhibitory effects of 1 nM Ry are not seen in the first 12 hours. Consistent with their known opposite actions on Ca^{2+} flux through the RyR, 1 nM Ry and 10 μM Ry had contrasting effects on the long-term function of gonadotropes. Blocking Ca^{2+} release from Ry-sensitive ER with 10 μM Ry resulted in an accumulation of stored GTH-II over 24 hours. This response was preceded by a transient decrease in GTH-II secretion and production seen at 12 hours. These data suggest that ER $[Ca^{2+}]_L$ is involved in the control of long-term secretion, storage and production of GTH-II in this cell type. Additional studies, in which $[Ca^{2+}]_L$ is measured in specific subcompartments of the ER over prolonged periods of time, are clearly required.

Although acute GnRH-stimulated GTH-II release is mediated by RyR, 1 nM Ry treatment did not mimic the effects of long-term GnRH receptor activation. Specifically, both sGnRH and cGnRH-II stimulated GTH-II release over 24 hours and did not significantly affect cellular GTH-II contents, but direct continual activation of RyR led to decreases in these parameters. However, it is important to note that GnRH receptor activation, in contrast to 1 nM Ry treatment, would be expected to deplete Ry-sensitive Ca^{2+} stores only transiently, allowing them to refill after the initial signal. Therefore, it is possible that long-term GnRH signalling involves a combination of RyR activation with inactivation. Alternatively, sGnRH and cGnRH-II may activate additional signalling systems that help maintain long-term GTH-II secretion and cellular contents. This latter alternative seems likely since long-term GnRH and 1 nM Ry treatments both affected GTH-II β subunit mRNA levels in a similar fashion (see Discussion below).

GnRH-stimulated GTH-II release (at 2 hours) is also known to require PKC (reviewed in Chang et al., 1996; Chapter 2). Previous studies have indicated that the PKC activator, TPA, decreases GTH-II cellular contents by ~40% after 6 hours; significant hormone release was elicited during the same period (Jobin et al., 1993). Interpretation of these data is difficult because long-term treatment with phorbol esters downregulates cellular PKC levels, in addition to the initial activation of the kinase. Although acute GnRH signalling does not involve PKA, agents that stimulate the cAMP/PKA pathway potentiate GnRH-stimulated GTH-II release (reviewed in Chang et al., 2000). Our data indicate that, like GnRH, cAMP-mobilizing agonists (such as forskolin) are also effective stimulators of long-term GTH-II secretion. However, forskolin also reduced cellular GTH-II contents, despite increasing total GTH-II production. Thus, the factors that regulate the long-term cellular GTH-II levels remain to be elucidated. Since goldfish gonadotropes store relatively small amounts of mature (immunoreactive) GTH-II, compared to the amount released over 24 hours, the regulation of glycoprotein processing and other post-translational steps may be important and possibly rate-limiting over long periods of stimulation.

Interestingly, the results of the present experiments with CPA and BHQ, together with previous observations (Johnson et al., 2000), provide physiological evidence that multiple intracellular Ca^{2+}ATPases can be pharmacologically differentiated in this cell type. If this is the case, isoform specific SERCA inhibitors would be powerful tools in functional studies of multiple intracellular Ca^{2+} stores. Recent evidence suggests that subtle structural changes in SERCA proteins can dictate drug activity. Specifically, single amino acid substitutions on SERCA (at Phe^{256}) confer Tg-resistance to Chinese hamster lung fibroblast cell lines (Yu et al., 1998). However, the pharmacological phenomenon of apparent selectivity between SERCA inhibitors is probably cell type-specific, as all SERCA inhibitors have identical short- and long-term actions in goldfish somatotropes (Johnson and Chang, 2000b; JD Johnson and JP Chang, unpublished data).

Positive and negative regulators of GTH-II β-subunit gene expression

Results from this study also provide information on the regulation of GTH-II gene expression by multiple Ca^{2+}-dependent mechanisms. We demonstrated that all three treatments designed to perturb intracellular Ca^{2+} stores modulated the levels of GTH-II β subunit mRNA, although with strikingly different kinetics. Inhibiting Ca^{2+} flux from RyR resulted in a rapid and transient increase in mRNA by 2 hours. In contrast, Tg and 1 nM Ry, treatments that would both elevate $[Ca^{2+}]_c$ and deplete $[Ca^{2+}]_L$, did not modulate GTH-II β subunit mRNA at this timepoint. Since 10 μM Ry does not acutely elevate $[Ca^{2+}]_c$ (JD Johnson and JP Chang, unpublished observations), we interpret these data as evidence for a novel signalling pathway, originating from a RyR-specific compartment of the ER lumen, which can be positively coupled to GTH-II gene expression. Although the exact nature of this pathway remains to be determined, it is not likely caused by elevated $[Ca^{2+}]_c$ or ER $[Ca^{2+}]_L$ depletion.

The findings that Tg and 1 nM Ry treatments caused opposite changes in GTH-II β-subunit mRNA levels by 12 hours provides further evidence of the specific control of GTH-II mRNA levels by Tg-sensitive stores and Ry-sensitive Ca^{2+} stores. Based on these findings with Ry, a hypothesis on how GnRH may act to regulate GTH-II gene expression can also be proposed. The temporal profile of the responses to 1 nM Ry (this study) agreed well with data on the *in vivo* time-dependent effects of sGnRH and cGnRH-II on GTH-II β subunit mRNA accumulation (Khakoo et al,. 1994), as well as results from similar on-going *in vitro* studies (C Klausen, JP Chang and HR Habibi, personal communication). All three treatments (1 nM Ry, 100 nM sGnRH and 100 nM cGnRH-II) did not affect GTH-II β subunit mRNA levels at early timepoints, but significantly elevated these values by 12 hours, followed by a suppression by 24 hours. Collectively, these findings suggest that GnRH and Ry may regulate GTH-II mRNA levels by a common mechanism. In addition, these data are consistent with the proposed involvement of RyR in GnRH signalling in goldfish gonadotropes. Interestingly, although the activation of VGCC is important for the maintenance of GnRH-stimulated secretion from goldfish gonadotropes over 2 hours, stimulating Ca^{2+} entry through VGCC with KCl resulted in a signal that was negatively coupled to

GTH-II mRNA levels over 12 hours. Therefore, it appears unlikely that VGCC are involved in the GnRH-stimulated increases in GTH-II β subunit mRNA seen over the same time period. Our results contrast with those reported for rat gonadotropes and related cell lines, where elevated $[Ca^{2+}]_c$ is positively coupled to GTH-II gene expression (Holdstock et al., 1996 and references therein).

Despite divergent actions at earlier timepoints, the net effect of all three intracellular Ca^{2+} store-modulating treatments was the reduction of GTH-II mRNA over 24 hours. Although rare in the literature, there is precedent for the inhibition of gene expression by Tg. For example, Tg treatment inhibits the transcription of the glucose-sensitive S14 gene of hepatocytes (Sudo and Mariash, 1996), although the exact mechanisms are not known. Tg evokes Ca^{2+} signals through a combination of Ca^{2+} efflux from intracellular stores, followed by a prolonged store-operated Ca^{2+} influx. Although the results with KCl certainly demonstrate that transient cytosolic Ca^{2+} signals can be negatively coupled to GTH-II mRNA accumulation, further experiments are required to directly examine whether the Ca^{2+}-store modulating agents used in the present study reduce GTH-II mRNA by depleting ER $[Ca^{2+}]_L$, increasing $[Ca^{2+}]_c$, or by other means.

Dissociation of the regulation of GTH-II mRNA and net GTH-II protein production

At first glance, the similarity of the Tg-induced decreases in GTH-II contents and GTH-II gene expression seem to suggest a causal relationship between the two. However, the observation that net GTH-II production is not altered over the course of the Tg treatment suggests a dissociation between the regulation of mRNA levels and protein production. Part of these differences can be accounted for by the expected delay between changes in mRNA expression and the appearance of the mature protein. Perhaps there is a substantial pool of immature, and therefore non-immunoreactive, GTH-II or (its subunit monomers) in the cells. However, a simple delay between changes in mRNA levels and alterations of protein contents is insufficient to explain the results observed following 10 μM Ry treatment. Collectively, these data provide a strong basis to propose that levels of mRNA and protein represent indices of largely independent cellular functions in goldfish gonadotropes.

Summary and physiological significance

Goldfish gonadotropes are controlled by a multitude of neuroendocrine regulators, suggesting the potential for agonist-specific control of GTH-II secretion and production. Indeed, GTH-II secretion and mRNA expression are controlled in an agonist-specific manner during some parts of the annual reproductive cycle (e.g. Khakoo et al., 1994), possibly through neuropeptide-specific Ca^{2+} signalling systems (Johnson et al., 2000; Johnson et al., 1999; Chapters 4, 5, 8). We have demonstrated that multiple components of the extended secretory pathway, such as acute agonist-stimulated GTH-II release, basal GTH-II release, long-term GTH-II secretion, GTH-II cellular contents, net GTH-II production and GTH-II β subunit mRNA levels, can be controlled

independently. Signalling events linked to GnRH, Tg-sensitive Ca^{2+} stores and Ry-sensitive Ca^{2+} stores are differentially coupled to these cellular functions in goldfish gonadotropes. I hypothesize that function-specific Ca^{2+} stores may represent a general mechanism for the independent control of cellular functions by multiple agonists.

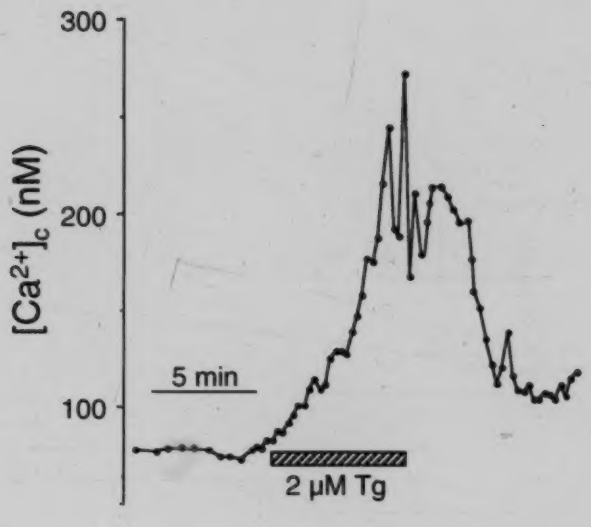

Figure 11.1. Tg-sensitive Ca^{2+} stores are present in goldfish gonadotropes. Treatment of morphologically identified goldfish gonadotropes with 2 μM Tg (*hashed bar*) for 5 minutes increased $[Ca^{2+}]_c$. The example trace is representative of 6 experiments on separate cultures.

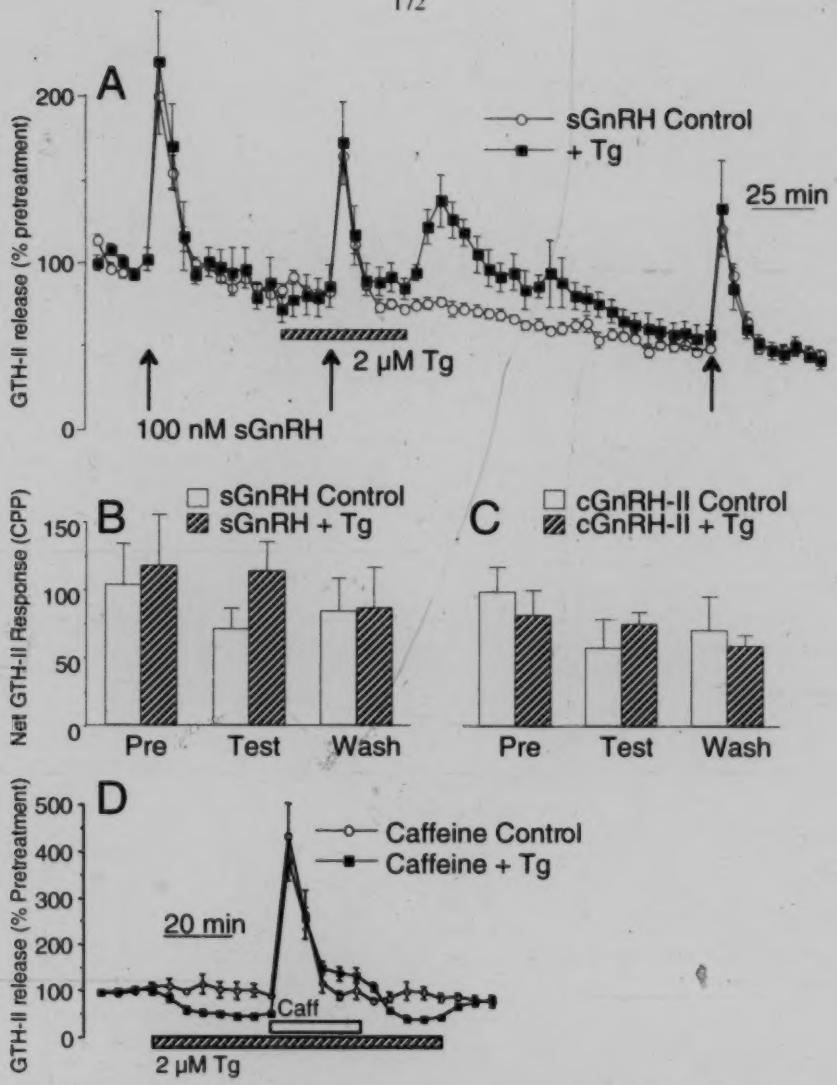

Figure 11.2. Tg-sensitive Ca^{2+} stores are not involved in acute GnRH-stimulated GTH-II release. (A) Dispersed pituitary cells were challenged 3 times with 5-minute pulses of 100 nM sGnRH (*arrows*). 2 μM Tg was present (*hashed bar*) during the 2nd pulse in treatment columns. Net GTH-II release responses to sGnRH (B) and cGnRH-II (C) are quantified (area under the curve) as described in Chapter 3. Units are cumulative % pretreatment. Treatment pulses were not different from sequential or parallel controls. (sGnRH n = 12; cGnRH-II n = 12). D) 2 μM Tg (*hashed bar*) did not reduce the acute GTH-II release response to 10 mM caffeine (*white bar*; n=8).

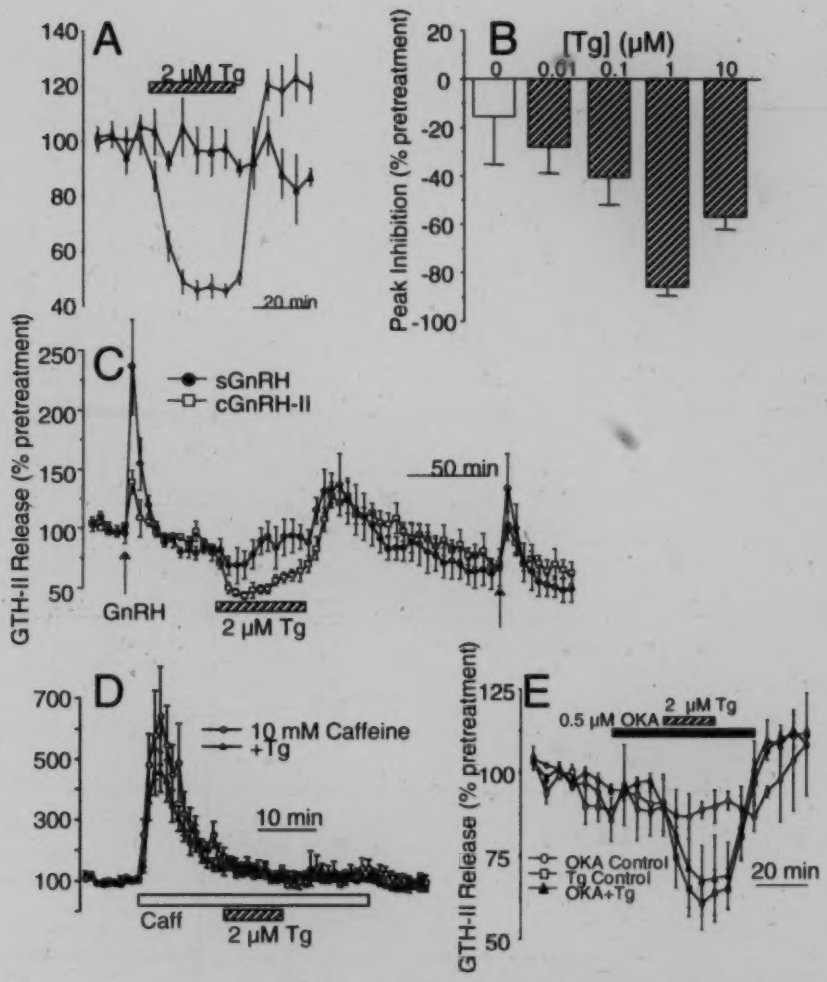

Figure 11.3. Depletion of Tg-sensitive stores reduces basal GTH-II release. A) Treatment with 2 µM Tg (*hashed bar*) rapidly reduced basal GTH-II release compared to untreated cells (n = 10). B) Dose-response characteristics were quantified from cell column perifusion experiments. Net GTH-II release, relative to the pretreatment, was integrated during the duration of Tg treatment (10 minutes). Samples were collected every minute (n = 4). C) Pre-exposure to 100 nM sGnRH, but not 100 nM cGnRH-II, for 5 minutes (*arrows*) prevented the Tg-induced decrease in GTH-II release. Protocol is identical to that of Figure 11.2 A, except that 2nd pulse was omitted. (n = 10). D) Tg did not suppress GTH-II release in the presence of 10 mM caffeine (n=6). E) 0.5 µM okadaic acid (*black bar*) did not effect the decrease in GTH-II release elicited by 2 µM Tg (*hashed bar*; n = 4).

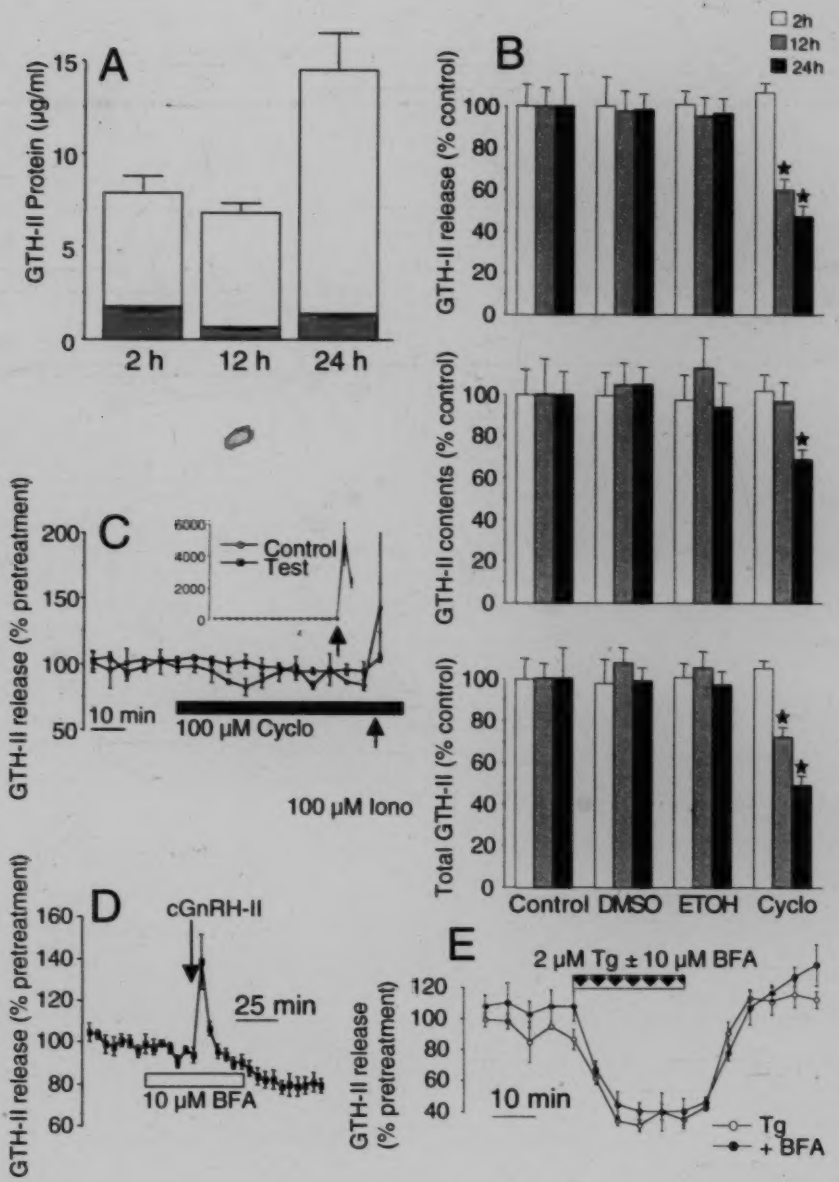
A
GTH-II Protein (µg/ml)
15
10
5
0
2 h
12 h
24 h
B
2h
12h
24h
GTH-II release (% control)
GTH-II contents (% control)
Total GTH-II (% control)
100
80
60
40
20
0
Control
DMSO
ETOH
Cyclo
C
GTH-II release (% pretreatment)
200
150
100
50
6000
4000
2000
0
Control
Test
10 min
100 µM Cyclo
100 µM Iono
D
GTH-II release (% pretreatment)
160
140
120
100
80
60
cGnRH-II
25 min
10 µM BFA
E
GTH-II release (% pretreatment)
120
100
80
60
40
2 µM Tg ± 10 µM BFA
10 min
Tg
+ BFA

Figure 11.4. Basic characterization of long-term GTH-II release, storage and production. (A) Shown is a representative example of GTH-II release (*white bars*) and cellular contents (*gray bars*) from unstimulated goldfish pituitary cells over 2, 12 and 24 hours (n = 3). (B) Long-term incubation with 0.1% DMSO or 0.1% ETOH did not affect characteristics of GTH-II release, contents or net production (n = 8). However, 100 µM cycloheximide altered long-term GTH-II protein levels (n = 20). *Star* denotes significant difference from time-matched control cultures. (C) Treatment with 100 µM cycloheximide (*black bar*) did not reduce acute basal GTH-II release or GTH-II release evoked by 100 µM ionomycin (*arrow*). *Inset* displays the entire experiment showing the full extent of the ionomycin-stimulated GTH-II release response (n = 6). (D) Basal and evoked GTH-II release are not inhibited by 10 µM brefeldin A (*white bars*). Cells were challenged with 5-minute pulses of 100 nM sGnRH (not shown) or 100 nM cGnRH-II as indicated by *arrows*. GTH-II release responses were similar in control columns exposed to 3 sequential GnRH pulses in the absence of BFA (n = 4). (E) The decrease in basal GTH-II release evoked by 2 µM Tg was not affected by co-application of 10 µM BFA (*checkered bar*; n = 4).

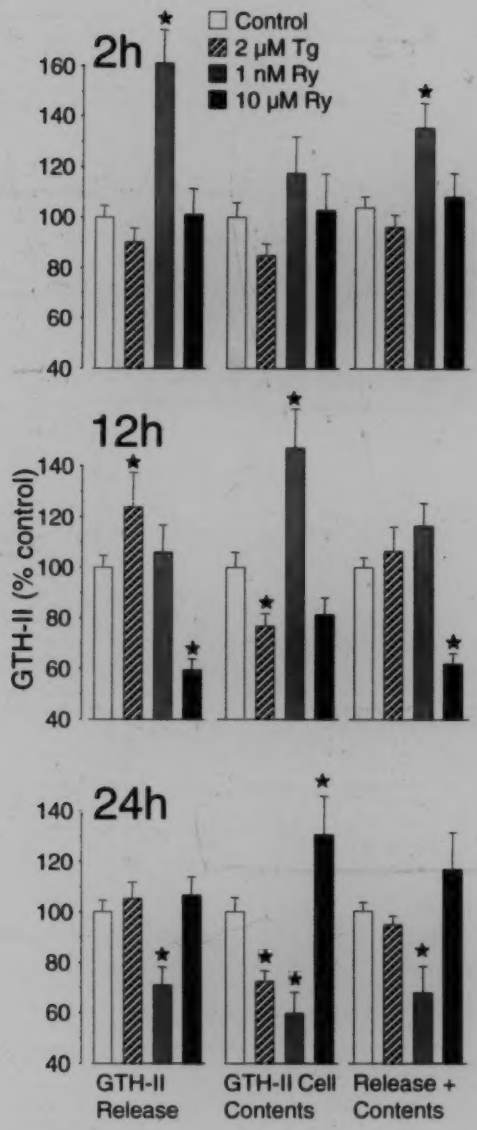

Figure 11.5. Tg and Ry differentially modulate long-term GTH-II release, cellular contents and net production after 2, 12 and 24 hours. Data were normalized as a percentage of untreated control cultures. *Star* denotes significant difference from time-matched control cultures (2 µM Tg, n=36; 1 nM Ry, n=12, 10 µM Ry, n=12).

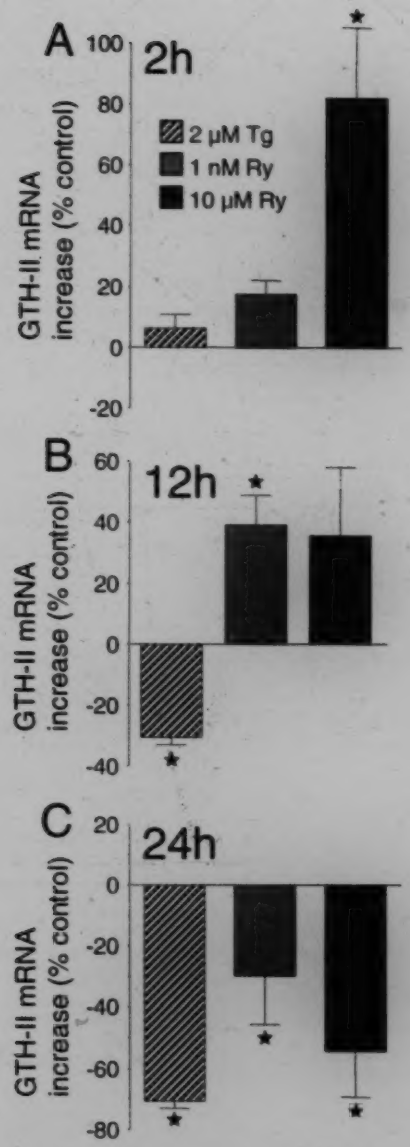

Figure 11.6. Tg and Ry differentially modulate long-term GTH-II β-subunit mRNA accumulation after 2, 12 and 24 hours. Northern blots were quantified using densitometry and normalized to the internal standard (18s ribosomal RNA). Data were expressed relative to time-matched untreated cultures. *Star* denotes significant difference from time-matched control cultures (n = 4).

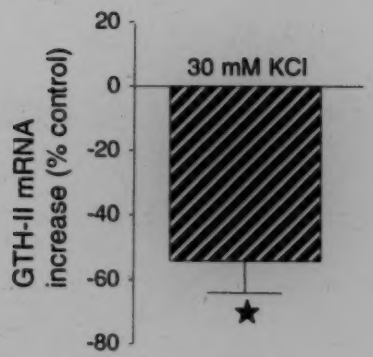

Figure 11.7. Cytosolic Ca^{2+} signals, mediated by extracellular Ca^{2+} influx through VGCC, reduces GTH β-subunit mRNA levels relative to untreated cells. Cells were exposed to 30 mM KCl for 30 minutes. GTH mRNA was measured after 12 hours and quantified as in Figure 11.6. *Star* denotes significant difference from time-matched control cultures (n = 4).

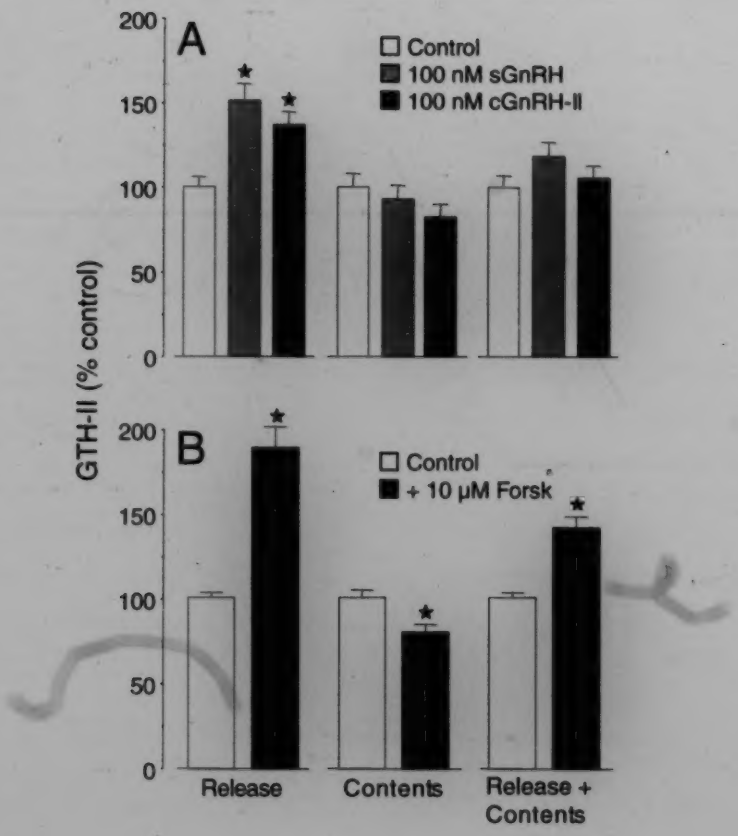

Figure 11.8. GnRH and cAMP modulate long-term gonadotrope activity. Cultures were treated with 100 nM sGnRH or 100 nM cGnRH-II (A; n = 12) or 10 µM forskolin (B; n = 12) for 24 hours. *Star* denotes significant difference from time-matched control cultures.

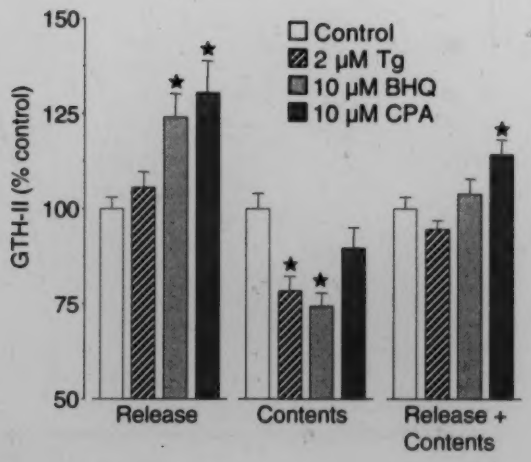

Figure 11.9. Differential effects of SERCA inhibitors on long-term gonadotrope activity. Cultures were treated with 2 μM Tg, 10 μM BHQ or 10 μM CPA for 24 hours (n = 12). *Star* denotes significant difference from time-matched control cultures.

Chapter 12.

Differential regulation of GH secretion, cellular contents and mRNA by multiple intracellular Ca^{2+} stores

The regulation of multiple components of the extended secretory pathway by Ca^{2+} may be complex (Chapters 1, 11). Multiple, function-specific Ca^{2+} stores offer a solution to the problems associated with the independent regulation of multiple cellular functions by Ca^{2+} (Johnson and Chang, 2000a). However, whether certain ER Ca^{2+} stores regulate exocytosis, while others control gene expression or protein processing is still poorly understood.

Results from previous chapters (Chapters 7, 8, 9) have shown that goldfish somatotropes likely possess multiple intracellular Ca^{2+} stores, but that Tg-sensitive Ca^{2+} stores do not participate in the control of GH release by sGnRH or cGnRH-II. In this chapter, the role(s) of Tg-sensitive Ca^{2+} stores in several other aspects of the extended secretory pathway was examined using Ca^{2+} imaging, radioimmunoassay measurements of hormone secretion and cellular GH content, and Northern analysis. The relative contribution of Tg-sensitive Ca^{2+} stores to the control of long-term GH secretion, storage, production and mRNA expression was compared to the role of separate Ry-sensitive Ca^{2+} stores.

Results

Uncoupling of Tg-sensitive Ca^{2+} stores from acute GH release

In the present study, I sought to confirm the presence of Tg-sensitive Ca^{2+} stores in single identified somatotropes and establish whether Ca^{2+} released from these pools can evoke GH secretion. In cells treated with 2 μM Tg, $[Ca^{2+}]_c$ increased slowly and steadily before gradually decaying to a higher baseline value (Fig. 12.1); Tg-stimulated Ca^{2+} signals are presumably initiated as a result of the uncompensated passive leak from SERCA-containing intracellular Ca^{2+} stores. Compared with Ca^{2+} signals evoked by Tg in the same cell, GnRH generated Ca^{2+} signals with distinct kinetics. Ca^{2+} signals evoked by 100 nM sGnRH (Fig. 12.1) or 100 nM cGnRH-II (data not shown) displayed a faster rate of rise and a rapid decay during the application period, consistent with a more 'active' release of Ca^{2+} from intracellular stores (Johnson and Chang, 2000b; Chapter 7). At the resolution used in the present study, the spatial pattern of Tg- and GnRH-evoked Ca^{2+} signals were not substantially different (Fig. 12.1).

In spite of the fact that 2 μM Tg elicited Ca^{2+} signals with similar maximal amplitudes when compared to endogenous secretagogues such as sGnRH and cGnRH-II (Fig. 12.1 and Fig. 12.2A; Johnson and Chang, 2000b; Chapter 7), Tg failed to stimulate the release of GH in 'rapid fraction' cell column perifusion experiments (Fig. 12.2B). In contrast, treating somatotropes with a depolarizing concentration of KCl (30 mM) in order to activate Ca^{2+} influx through VGCC, resulted in the expected positive relationship between Ca^{2+} signalling and GH release (Fig. 12.2C,D). Taken together, these data suggest that Ca^{2+} signals generated by 2 μM Tg may be selectively uncoupled from the acute regulation of GH secretion in goldfish somatotropes.

The inability of Tg treatment to cause acute GH release was also confirmed at several additional doses below 2 μM (Fig. 12.3). Although Tg is widely considered to be specific at doses ranging from subnanomolar up to 1 μM (ED_{50}: 2 nM, Vermeer et al., 1990; ~50 nM, Lytton et al., 1991), its specificity has not been rigorously established at higher concentrations (i.e. ≥ 10 μM). GH release slowly increases in cells treated with 10 μM Tg (Fig. 12.3). Given that this GH release response is 1) only seen at very high doses of Tg, 2) has peculiar kinetics and 3) does not follow a typical sigmoidal dose-response relationship, one may speculate that it results from non-specific or secondary actions of Tg. Perhaps GH is expelled from the ER after a threshold of $[Ca^{2+}]_L$ is reached. All subsequent experiments were performed using 2 μM Tg, a dose at which Ca^{2+} signals have been characterized.

Characterization of basal GH release and storage

In preparation for studies examining the effects of selective manipulations of Ca^{2+} stores on the long-term regulation of basal GH secretion and storage, we first characterized some features of these parameters in unstimulated goldfish somatotropes. Cellular GH contents of unstimulated cells, maintained in testing medium, remained stable for at least 24 hours (results from a representative experiment are shown in Fig. 12.4A). Similarly, the rate of basal GH secretion did not change significantly between the timepoints (2 hours: 370 ± 97 ng/hour; 12 hours: 263 ± 62 ng/hour; 24 hours: 271 ± 52 ng/hour; pooled results from 3 separate experiments). Although the absolute amount of GH stored and released varied between cell cultures, the relative proportion of these two components was similar in different experiments. For this reason, GH values were normalized with respect to controls in subsequent analysis.

Endocrine cells are known to release proteins, including hormones, through multiple routes, including the regulated secretory pathway and the constitutive secretion pathway (Kelly, 1985). To determine the relative involvement of these secretory pathways in somatotropes, the effects of cycloheximide on basal GH secretion and cellular contents were monitored. Inhibition of protein synthesis with cyclohexamide (100 μM) did not reduce GH release in 2-hour static incubation studies (Fig. 12.4B). Likewise, long exposures of cyclohexamide did not affect basal GH release in perifusion experiments (Fig. 12.4C). These results suggest that the constitutive secretory pathway does not play a role in basal GH secretion (up to 2 hours) from goldfish somatotropes. The observation that exocytosis evoked by ionomycin-mediated Ca^{2+} influx is also unaffected by cyclohexamide, indicates that somatotropes do not preferentially release newly synthesized GH through the regulated secretory pathway.

Although prolonged treatment (12 and 24 hours) with cyclohexamide produced the expected decrease in cellular GH contents, a paradoxical increase in GH contents was observed after 2 hours (Fig. 12.4B). At this time, we can only speculate as to the possible underlying mechanism of this response. Perhaps cycloheximide inhibited the formation of a certain pool of proteins with rapid

turnover/synthesis rates which are normally responsible for the degradation of some stored GH.

Long-term actions of Tg- and Ry-sensitive Ca^{2+} stores on the GH secretory pathway

Having ruled out a direct coupling to acute GH release, we asked whether Tg-sensitive Ca^{2+} stores regulate other aspects of the secretory pathway, such as long-term GH release, cellular GH contents, total GH (release + contents; an index of production) and GH mRNA levels at 3 time-points. In addition, we compared the effects of Tg with Ry at stimulatory and inhibitor doses.

Over all, the long-term effect of Tg treatment (2 μM) was the release of GH from the secretory pathway, without a concomitant maintenance of cellular GH contents (Fig. 12.5). In contrast, activation of RyR with 1 nM Ry produced a biphasic response. In the first 2 hours, Ca^{2+} release form Ry-sensitive store is positively coupled to GH release, contents and net production, whereas the net effect over 24 hours was a decrease in all of these parameters. On the other hand, blocking RyR Ca^{2+}-release channels with 10 μM Ry transiently elevated GH cellular contents and caused a slight increase in production. Further differences in the functional coupling of Tg-sensitive Ca^{2+} stores and Ry-sensitive Ca^{2+} stores to the extended GH secretory pathway are revealed when the data are examined at each timepoint. After 2 hours, Tg treatment had initiated a sustained GH release response. Although 1 nM Ry also stimulated GH release over 2 hours, this was accompanied by an increase in cellular GH contents. In contrast, the inhibition of these stores with 10 μM Ry increased GH contents without affecting secretion. At the 12 hour timepoint, Tg-treated cells continued to release stored GH without an apparent compensatory increase in hormone production. In the presence of Tg, cellular GH contents were reduced to a level similar to that seen in experiments with cyclohexamide. In contrast, both doses of Ry did not affect GH secretion and contents over 12 hours. Over 24 hours, the increase in GH release from Tg-treated cultures, although still significant, was at its lowest levels compared to controls. It is important to note that since our measurements are cumulative, these later determinations of GH release are still 'contaminated' with the high responses seen after 2 hours. Over the course of the entire experiment, the stimulatory dose of Ry had resulted in a reduction in basal GH secretion, presumably due to the failure to compensate with increases in GH contents and production. Nevertheless, the modest reduction in stored GH seen in 1 nM Ry-treated cultures was significantly different from the more robust decrease seen in Tg-treated cell during the same period ($P < 0.05$). On the other hand, blocking RyR had no significant effect on these parameters. Taken together, these data strongly suggest that manipulations of Tg-sensitive Ca^{2+} stores and Ry-sensitive Ca^{2+} stores in goldfish somatotropes differentially controls the long-term secretion and replenishment of GH.

Next, the role of these two intracellular Ca^{2+} stores in the control of GH gene expression was assessed by Northern blot. Neither 2 μM Tg nor 1 nM Ry significantly altered GH mRNA levels at any of the timepoints tested (Fig. 12.6), in spite of their ability to evoke robust GH secretion during the first 2 hours. Surprisingly, application of the blocking dose of Ry (10 μM) resulted in a

significant, but transient, elevation in GH gene expression at 2 hours, a time at which cellular GH contents and GH production were also increased. These results with 10 μM Ry suggest that perturbing Ry-sensitive Ca^{2+} stores, by blocking Ca^{2+}-release channels, can regulate GH biosynthesis. Although all of the treatments used in the present study were shown to modulate the apparent storage and production of GH, only the transient (2 hours) effects of 10 μM Ry are correlated with a change in GH mRNA levels.

Negative coupling of $[Ca^{2+}]_c$ to GH gene expression

In order to further evaluate the functional importance of specific Ca^{2+} signals, the role of $[Ca^{2+}]_c$ signals generated independently of intracellular Ca^{2+} stores in the regulation of GH gene expression needed to be addressed. It has previously been shown that KCl-stimulated Ca^{2+} signals in populations of goldfish pituitary cells are completely dependent on the influx of extracellular Ca^{2+} (Jobin and Chang, 1992). Thus, cells were treated with 30 mM KCl for 30 min to activate VGCCs and GH mRNA were measured 12 hours later. GH mRNA levels were reduced as a result of VGCC-mediated Ca^{2+} signals (Fig. 12.7). This result suggests that elevated $[Ca^{2+}]_c$ initiates a signal that is inhibitory to GH gene expression in the goldfish.

Discussion

Uncoupling of Tg-stimulated Ca^{2+} signals from acute GH release

I have hypothesized that cells use function-specific Ca^{2+} stores to generate a complex array of Ca^{2+} signals capable of controlling different components of the extended secretory pathway independently (Johnson and Chang, 2000a; Chapter 1). A prediction of such a hypothesis is that Ca^{2+} signals from certain intracellular stores would fail to modulate certain Ca^{2+}-dependent processes. The present study clearly indicates that such functional specificity occurs. No GH release was seen at a dose of Tg which evoked increases in $[Ca^{2+}]_c$ of similar maximal amplitude as those generated by GnRH, suggesting that one class of ER Ca^{2+} stores is functionally uncoupled from the acute control of exocytosis. Different degrees of uncoupling between Ca^{2+} signals and hormone release have been reported for cell lines derived from the mammalian pituitary (Trueta et al., 1999). Data reported in Chapter 7 indicate that Tg-sensitive Ca^{2+} stores do not mediate the acute control of GH release by endogenous Ca^{2+}-mobilizing agonists (sGnRH and cGnRH-II; Johnson and Chang, 2000b), in contrast to many other cell types (Berridge, 1997; Tse et al., 1997). Instead, acute GnRH-stimulated GH release depends on a rapidly-depleting intracellular Ca^{2+} pool that is sensitive to TMB-8 and caffeine (Johnson and Chang, 2000b; Chapter 7). In addition, GH release evoked by sGnRH, but not cGnRH-II also appears to involve an IP_3R component; conversly cGnRH-II-stimulated GH release is selectively mediated by RyR during some times of the year (Chapter 8).

Tg-stimulated $[Ca^{2+}]_L$ depletion induces the prolonged release of GH from the secretory pathway

What is the role of Tg-sensitive Ca^{2+} stores in goldfish somatotropes, if not to directly regulate acute GH release or mediate agonist signalling? Results presented herein indicate that the depletion of Tg-sensitive ER Ca^{2+} stores led to a long-term emptying of stored GH. This response was not accompanied by a compensatory increase in GH production (assessed by net hormone present or GH mRNA levels). The net result was a reduction in the cellular GH contents. Although the decrease in cellular GH contents induced by Tg is quantitatively similar to that seen in cyclohexamide-treated cells, the observation that Tg does not affect GH production or mRNA levels suggests that Tg and cycloheximide have different mechanisms of action. Therefore, Tg must reduce cellular contents at a site which is distal to protein synthesis on the extended secretory pathway. Although the exact mechanism of Tg action remains to be determined, I hypothesize that Tg accelerates GH traffic through the distal parts of the secretory pathway. In light of the findings that 2 μM Tg does not directly stimulate acute exocytosis, we propose that the prolonged secretion of GH in 2 μM Tg-treated cultures results from an increased amount of GH released through the 'basal' mechanism. Since GH is not normally released through a constitutive secretory pathway, our findings are consistent with the hypothesis that GH release is enhanced by increasing the rate at which a pool of GH secretory granules (of the regulated secretory pathway) becomes 'releasable'. In some endocrine cell types, basal secretion has been attributed to a portion of secretory granules which undergo random fusion in the steady state (Varro et al., 1996). However, these data cannot rule out the possibility that some GH may be shunted into a constitutive, or constitutive-like secretory pathway, in Tg-treated cells. We are not aware of studies, in other model systems, which have measured similar reductions in hormone contents in Tg-treated cells. It is likely that the actions of Tg may be cell type-specific. Studies of goldfish gonadotropes, in which Tg also evokes a reduction in cellular hormone contents, have indicated that Tg-sensitive Ca^{2+} stores play an important role in the long-term regulation of GTH-II mRNA levels (see Chapter 11).

Inhibition of Ry-sensitive Ca^{2+} stores transiently stimulates GH production

It has been reported that Tg-mobilizes only a fraction of stored Ca^{2+}, equivalent to between ~40% and 60%, depending on the cell type (Pizzo et al., 1997; Gamberucci et al., 1995; Tanaka and Tashjian, 1993). Thus, we examined whether other components of the ER are functionally important in somatotropes. Previous studies have shown that Ry-sensitive Ca^{2+} stores participate in acute agonist Ca^{2+} signalling in mammalian somatotropes (Herrington and Hille, 1994; Petit et al., 1999). In spite of fact that RyR is found in many tissues (Sorrentino and Volpe, 1993), the modulation of gene expression and protein synthesis by Ry-sensitive Ca^{2+} stores is not well characterized in non-muscle cells. Results of the present study provide, for the first time, evidence of the involvement of RyR in the long-term functioning of somatotropes. Stimulation of ER Ca^{2+} release with 1 nM Ry evoked significant GH secretion after 2 hours. This response was

transient and eventually resulted in the inhibition of GH release over 24 hours, unlike the prolonged secretory activity of Tg. On the other hand, the inhibitory dose of Ry did not affect the release of GH. Collectively, these data suggest that GH release is positively couple to Ca^{2+} release from Ry-sensitive stores, at least initially.

Interestingly, whereas both Ry treatments elevated GH contents at 2 hours, only 10 μM Ry increased GH mRNA. This result, suggests (indirectly) that part of the increase in total GH seen in cells treated with 1 nM Ry over 2 hours results from some post-transcriptional enhancement of GH production. On the other hand, the increased GH contents in 10 μM Ry-treated cells can be accounted for by the significant increase in GH mRNA expression. It can be proposed that the inhibition of normal Ca^{2+} homeostasis, and perhaps an elevated $[Ca^{2+}]_L$, rather than a cytosolic Ca^{2+} signal, mediates acute changes in GH gene expression. Although others have elegantly shown the importance of ER $[Ca^{2+}]_L$ in the control of ER regulatory genes (Llewellyn et al., 1996), this is the first time such a mechanism has been considered as a primary regulator of hormonal mRNA in cultured pituitary cells. A complete test of this hypothesis awaits the measurement of $[Ca^{2+}]_L$ in the Ry-sensitive ER of intact somatotropes. Nevertheless, our results clearly demonstrate that ER Ca^{2+} stores sensitive to Ry, but not Tg, can be coupled to GH mRNA expression. This finding does not rule out the possibility that Tg-sensitive Ca^{2+} pools control the expression of other genes in somatotropes. Indeed, the independent modulation of multiple gene sets would offer additional degrees of Ca^{2+} signalling specificity to the cell.

Role of $[Ca^{2+}]_c$ in GH production

We directly examined the relationship between $[Ca^{2+}]_c$ and GH mRNA expression using depolarizing concentrations of KCl to generate Ca^{2+} signals mediated by VGCC (but presumably not intracellular stores). Surprisingly, Ca^{2+} influx through VGCC strongly inhibited GH mRNA expression, although they were positively coupled to GH release. In other experiments, ionomycin, which is also a potent GH secretagogue, dose-dependently decreased GH cellular contents and production (M Volk and JP Chang, unpublished results). Thus, these effects of KCl on goldfish somatotropes are not likely due to the preferential signalling by VGCC-mediated Ca^{2+} signals, as other have reported for some neurons and pituitary cells (Bading et al., 1993; Mulvaney et al., 1999), but may be generalized to elevation of $[Ca^{2+}]_c$. A comparable dissociation between GH release and GH synthesis has been noted in studies of KCl-treated mammalian somatotropes. Treating rat somatotropes with high [KCl] for 1 hour had no effect on GH mRNA levels, despite a robust secretory response (Barinaga et al., 1985). Similarly, some treatments that would be expected to increase cellular Ca^{2+} dramatically, such as replacing Ca^{2+}-free media with high Ca^{2+} media, fail to stimulate increases in GH mRNA expression in GH_3 cells and normal rat somatotropes (although prolactin synthesis was stimulated in GH_3 cells; White et al., 1981; Gick and Bancroft, 1985).

The findings that elevated $[Ca^{2+}]_c$ is negatively coupled to mRNA expression are compatible with the observed differential effects of 1 nM Ry and

10 μM Ry on GH mRNA levels. The major presumed difference between the two Ry treatments is that only the stimulatory dose activates the RyR to release Ca^{2+}, while both treatments would be expected to perturb the normal cycling of Ca^{2+} through the Ry-sensitive component of the ER. One possible interpretation of these findings is that 1 nM Ry generates two Ca^{2+} signals, one lumenal and one cytosolic, which have opposing effects on GH mRNA levels, thus resulting in no net change in GH mRNA accumulation. Additional work is needed to establish signalling cascades coupling Ry-sensitive Ca^{2+} stores to GH gene expression.

Future studies will also be required to determine whether this unique relationship between $[Ca^{2+}]_c$ and GH mRNA plays a role in the long-term signalling of the many endogenous agonists and antagonist that regulate the function of goldfish somatotropes. sGnRH, cGnRH-II, dopamine and PACAP are all known to require VGCC activation to sustain long-term (2 hours) evoked GH release (Chang et al., 2000). Recent preliminary experiments have shown that sGnRH and cGnRH-II stimulated GH mRNA accumulation, over the same time period when it was inhibited by KCl (C Klausen, JP Chang, HR Habibi, personal communication). Therefore, it seems likely that VGCC-independent post-receptor pathways are important in the long-term effects of GnRH on GH gene expression.

Summary and Physiological Significance

The fact that goldfish somatotropes are regulated by multiple agonists suggests that functional specificity of Ca^{2+} signalling has to be sufficiently complex in order to ensure the precise independent control of specific cellular functions by specific extracellular signals. Multiple intracellular Ca^{2+} stores may impart such complexity and specificity. Future studies should include the examination of novel intracellular Ca^{2+} stores and more mRNA species involved in different aspects of the secretory pathway.

In conclusion, the present results provide new insights into the regulation of multiple aspects of the extended secretory pathway by two intracellular Ca^{2+} stores, and by cytosolic Ca^{2+} signals. Moreover, these data support a model whereby both $[Ca^{2+}]_L$ signals and $[Ca^{2+}]_c$ participate in the control of hormonal gene expression. I propose that function-specific Ca^{2+} stores may represent a novel mechanism for the independent control of cellular functions by multiple agonists.

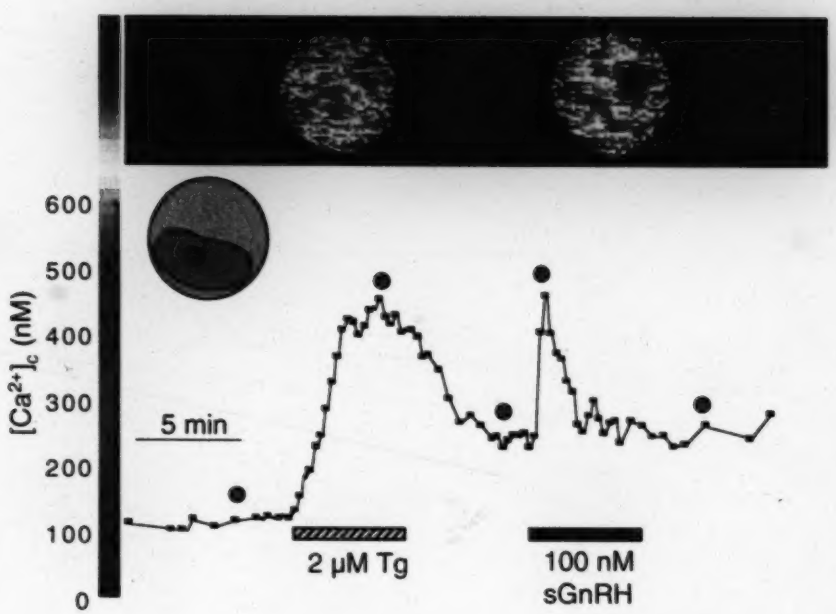

Figure 12.1. Comparison of Ca^{2+} signals evoked by Tg (2 µM) and sGnRH (100 nM) in morphologically-identified Fura-2 loaded somatotropes. Treatment duration is indicated by horizontal bars. *Top panel* shows false-colour images from selected times throughout the experiment (*gray dots*). A sketch of the cell is included (*inset*) for reference. Results are representative of cells from 7 independent cultures. Amplitude of Ca^{2+} signals evoked by 100 nM cGnRH-II are similar to those induced by Tg and sGnRH (not shown; Johnson and Chang, 2000b).

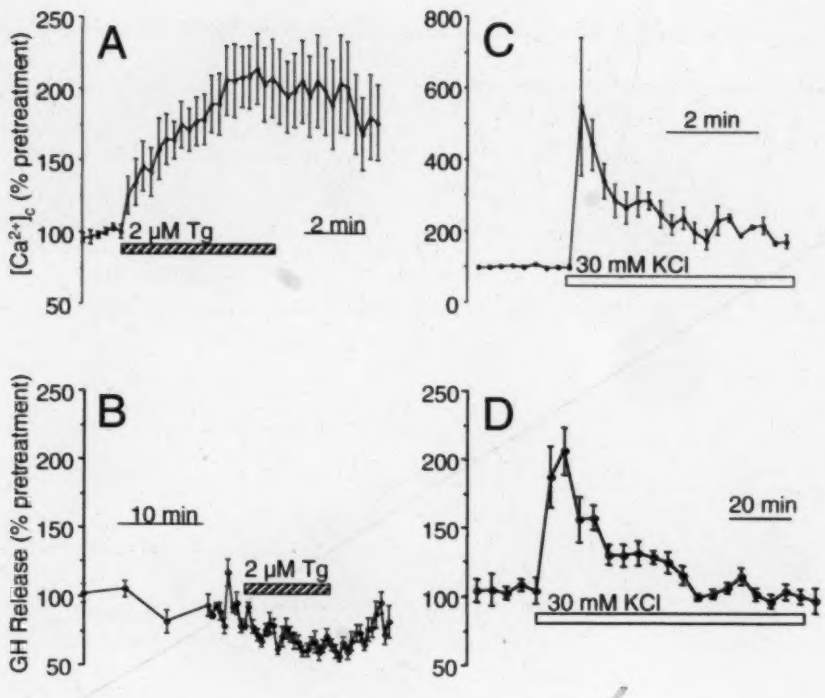

Figure 12.2. Comparison of GH release response and Ca^{2+} signals generated by Tg or 30 mM KCl. Treatment duration is indicated by horizontal bars. (A) Shown are pooled results of Ca^{2+} signals from ten 2 µM Tg-treated somatotropes from independent cultures. (B) Tg (2 µM), given under similar conditions failed to stimulated GH release from populations of somatotropes contained in mixed pituitary culture. Samples from the cell column perifusion were collected at 30 seconds where indicated (n = 5). (C) Activation of VGCCs with 30 mM KCl evoked biphasic Ca^{2+} signals in identified somatotropes. Pooled results from 6 cells from different cultures are shown. (D) 30 mM KCl stimulated GH release (n = 6).

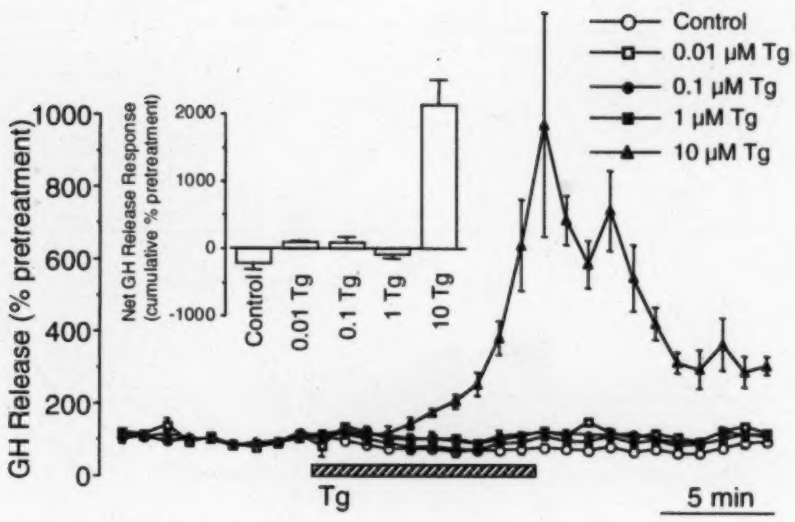

Figure 12.3. Dose-response relationship of Tg on GH secretion. Different concentrations of Tg were added to the perifusion as indicated (*Hashed bar*). Net responses, during the treatment period, are displayed in the *Inset*. (n = 4)

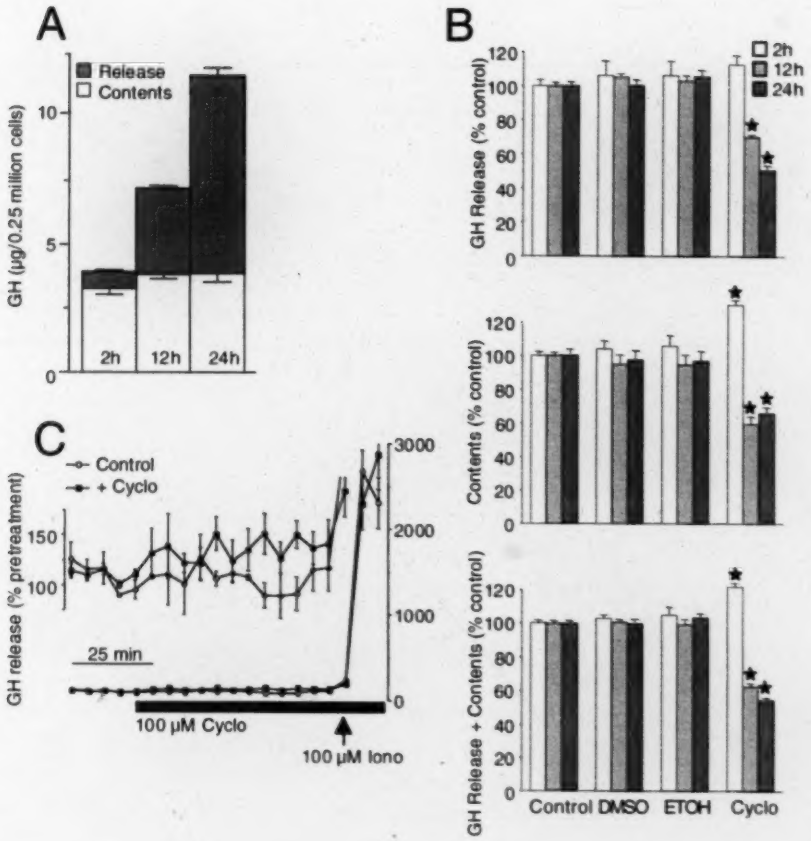

Figure 12.4. Characterization of long-term basal GH release and storage. (A) Mixed populations of dispersed pituitary cells release GH and maintain stable GH contents over 24 hours in testing media. A representative examples of 3 separate experiments is shown. (B) Long-term GH release and storage is not affected by 0.1% DMSO or 0.1% ETOH (n = 8). Treatment with cyclohexamide (100 µM) significantly reduced GH production and contents by 12 hours, after an initial stimulation seen at 2 hours (n = 20). (C) Extended treatment with 100 µM cyclohexamide (*black bar*) did not reduce basal or 100 µM ionomycin-stimulated GH release in perifusion experiments (n = 6). *Star* indicates significant difference compared with time-matched controls.

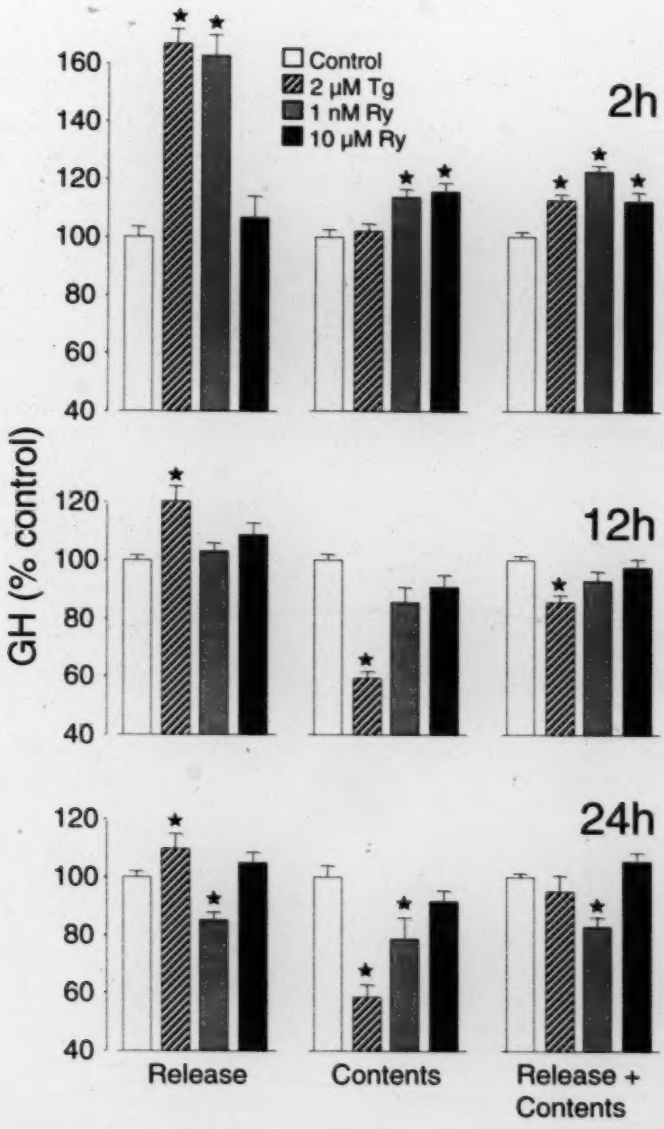

Figure 12.5. Differential coupling of ER Ca^{2+} stores sensitive to Tg or Ry to GH secretion, contents and production (secretion + contents) at 3 timepoints. GH values from cultures treated with 2 μM Tg (n = 36), 1 nM Ry (n = 16) or 10 μM Ry (n = 16) are normalized to time-matched controls (n = 48). See Figure 12.4 for typical absolute GH values (pre-normalization). *Star* denotes significant difference from time-matched controls.

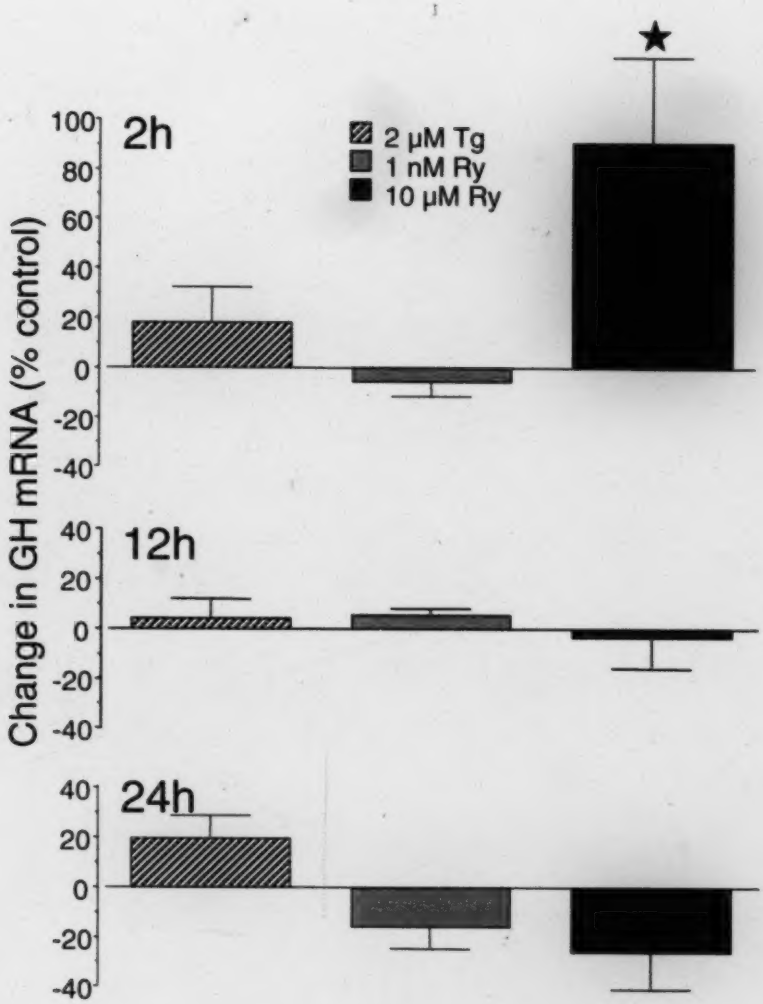

Figure 12.6. Tg and Ry differentially affect GH mRNA levels. Results are presented as relative change from untreated cultures at each time point (n = 4). GH mRNA was quantified using densitometry and normalized to an internal standard (18 s ribosomal RNA). *Star* denotes significant difference from time-matched control cultures.

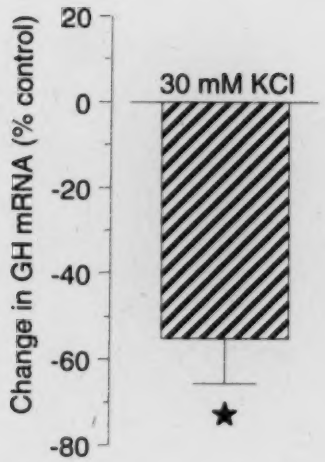

Figure 12.7. Cytosolic Ca^{2+} signals, mediated by extracellular Ca^{2+} influx through VGCC, reduce GH mRNA relative to untreated cells. Cells were exposed to 30 mM KCl for 30 minutes. GH mRNA was measured after 12 hours and quantified as in Figure 12.6. (n = 4). *Star* denotes significant difference from control.

Chapter 13.

Agonist- and function-specific Ca^{2+} signalling. Evidence from goldfish pituitary cells.

This thesis presents novel evidence that multiple intracellular Ca^{2+} stores play important roles in the physiology of neuroendocrine cells. Using both gonadotropes and somatotropes of the goldfish pituitary, I have conducted the first comprehensive, physiological study, in any endocrine cell type, that accommodates the theoretical complexity of Ca^{2+} signalling mediated by multiple (at least 5) intracellular Ca^{2+} stores. This study is also unique because the effects of multiple Ca^{2+} stores is assessed on multiple Ca^{2+}-sensitive components of the extended secretory pathway.

A key component of the thesis was the review of state-of-the-art concepts in the cell biology and biophysics of Ca^{2+} signalling. It is the first comprehensive review to integrate the current knowledge of the multiplicity of Ca^{2+}-dependent cellular functions and Ca^{2+} handling organelles with the complexity of strategies used by cells to encode information using Ca^{2+} signals. It was concluded that Ca^{2+} signalling must be complex in order to regulate multiple cellular functions. Moreover, the literature revealed that cells are likely to possess multiple functionally heterogeneous intracellular Ca^{2+} stores which generate complex Ca^{2+} signals capable of controlling the plethora of Ca^{2+}-dependent physiological endpoints. Novel hypotheses which had not previously been incorporated into the study of the neuroendocrine control of hormone release were proposed, and these became the basis for the experimental studies described in this thesis. Thus, Chapter 1, in its own right, represents an important contribution to the understanding of Ca^{2+} signalling, especially as it may pertain to neuroendocrine systems.

The major experimental findings can be summarized as follows. Ca^{2+} signals of sGnRH and cGnRH-II were analyzed, quantitatively, and found to be different from each other in gonadotropes. Complementing previous work, we provided the first direct evidence that two closely related endogenous neuropeptides activate partially independent suites of intracellular Ca^{2+} stores, suggesting that the agonist-specific Ca^{2+} signals are generated by agonist-specific Ca^{2+} stores. Since the available data indicate that sGnRH and cGnRH-II act through a single population of receptors on gonadotropes (i.e. no known receptor recognizes sGnRH but not cGnRH-II), the present findings provide further evidence for a novel form of post-receptor signalling complexity which, in this case, leads to the differential control of gene expression (Khakoo et al., 1994), and acute hormone release (Chapter 10).

Moreover, the apparent properties of the Ca^{2+} stores mediating acute GnRH-stimulated hormone release are different between gonadotropes and somatotropes. This included a parallel comparison of the mechanisms of action of caffeine between the two cell types. Experiments with Ry showed that, in addition to agonist-specificity (the first such evidence for somatotropes) and cell-type-specificity, there was a differential involvement of RyR based on the reproductive stage. This is the first study to demonstrate, using ryanodine, that pituitary RyR

mediates GnRH-stimulated hormone release, in any model system. In addition, these findings provide novel evidence of plasticity in the specific coupling of Ca^{2+} stores to evoked pituitary hormone release. The large data set that reveals the seasonal variations in caffeine-stimulated GTH-II and GH release represents the most comprehensive seasonal study of Ca^{2+}-dependent stimulus-secretion coupling to date, and strongly suggests that this mechanism of cellular plasticity may be extended to other intracellular Ca^{2+} stores.

Next I demonstrated that mitochondrial Ca^{2+} uptake is an active, albeit minor, regulator of basal Ca^{2+} homeostasis in somatotropes and proposed the existence of a sub-cellular Ca^{2+} circuit between the ER and the mitochondrial, as has been shown in other cell types (reviewed in Chapter 1; Duchen, 1999). Results from these investigations are the first to implicate the Ca^{2+} buffering activity of mitochondria in the acute control of hormone release under unstimulated conditions in any cell type. The selective involvement of mitochondrial Ca^{2+} buffering in sGnRH-, but not cGnRH-II-stimulated GH release provides additional evidence of the distinct signal transduction of these neuropeptides. These experiments were also interesting because they illustrated the principle that some Ca^{2+} signals can be strongly linked to exocytosis, in spite of their modest global amplitude.

The idea that Ca^{2+} signals can encode information independent of their amplitude was further supported by the observation that slow-rising Ca^{2+} signals, generated as a result of Tg treatment, did not stimulate hormone release. Since the major difference between these Ca^{2+} signals and those of secretagogues is the rate of rise, these data form the basis for the novel hypothesis that the rate of rise of Ca^{2+} signals can encode functionally relevant information in goldfish gonadotropes. In any case, this is one of the first clear demonstrations that Ca^{2+} signals from an intracellular Ca^{2+} store can be selectively uncoupled from a Ca^{2+}-dependent physiological function.

At this point, the results fulfilled many of the specific predictions and provided strong experimental evidence in support of several of the general hypotheses derived from Chapter 1. By virtue of their differential roles in basal and agonist-stimulated hormone release and Ca^{2+} homeostasis, several pharmacological classes of intracellular Ca^{2+} stores that co-exist and interact within gonadotropes and somatotropes were identified. The concept of function-specific Ca^{2+} stores was also supported by data suggesting the independent regulation of gene expression, as well as the long-term production, storage and secretion of hormones, by two pharmacological classes of Ca^{2+} stores. This is 1) the first study to examine the regulation of several aspects of the extended secretory pathway in the context of multiple Ca^{2+} stores in any cell type, 2) the first evidence that Ry-sensitive Ca^{2+} stores control the levels of hormone mRNA in a non-muscle cell, and 3) one of the only reports of negative coupling between Ca^{2+} signalling and gene expressionof a hormone. Collectively, the work described in this thesis suggests that the multiplicity of intracellular Ca^{2+} stores is of considerable physiological importance in pituitary cells.

Multiple functionally distinct Ca^{2+} stores

A minimal model that accounts for the data reported herein strongly suggest that both cell types may possess at least 4 non-mitochondrial Ca^{2+} stores. These findings have been integrated into new working models that describe aspects of the acute regulation of basal and agonist-stimulated hormone release in goldfish gonadotropes and somatotropes (Figs. 13.1, 13.2). Possible interactions with other signalling cascades, though only briefly dealt with in this thesis, are also incorporated into the model.

In goldfish gonadotropes, activation of secretion by both GnRHs requires TMB-8 sensitive Ca^{2+} stores. This seems to be the case in cells prepared from sexually regressed, as well as sexually mature, animals. The conditional agonist-specificity of the Ry-sensitive Ca^{2+} stores is the basis for assigning them as separate from the 'shared' GnRH- and TMB-8 sensitive pool. However, since the experiments involving TMB-8 were not done in parallel (on the same cell preps) as those using Ry, we cannot completely rule out the possibility that they represent the same store. In contrast, caffeine clearly stimulates GTH-II release by action on a completely independent class of Ca^{2+} stores that are depletion-resistant, RyR-independent and Tg-independent. Caffeine-sensitive Ca^{2+} stores are grouped together with IP_3-sensitive stores in this minimal model because both are selectively involved in sGnRH-signalling. Although caffeine is a known antagonist of the IP_3 receptor (Erhlich et al., 1994), direct experimental evidence linking the targets of caffeine and xestospongin C in goldfish gonadotropes is lacking. Moreover, the involvement of both caffeine- and IP_3-sensitive components in GnRH-stimulated GTH-II release has only been addressed in cells from sexually regressed fish. In contrast, Tg-sensitive Ca^{2+} stores are not involved in GnRH-stimulated GTH-II release at any stage. The significant divergence in both the spatio-temporal features, and the physiological outcomes, of Tg-evoked Ca^{2+} signals indicates that they originate from a Ca^{2+} pool independent from the others. Results from other experiments suggest that mitochondria may also be involved in Ca^{2+} homeostasis and hormone release in gonadotropes (CCCP increased GTH-II release by ~175% in static incubation experiments; increases could be blocked with BAPTA; JD Johnson and JP Chang, unpublished data). Although additional work is clearly required, the results of these investigations strongly support the hypothesis that goldfish gonadotropes contain at least 4 non-mitochondrial Ca^{2+} stores.

Similar logic was used to estimate that goldfish somatotropes also possess 4 non-mitochondrial Ca^{2+} stores. Ca^{2+} signalling and hormone release responses of both GnRHs are mediated by a TMB-8-sensitive Ca^{2+} pool, which is also sensitive to caffeine. Although Ca^{2+} release from RyR is sufficient to stimulate GH release, Ry-sensitive Ca^{2+} stores are only involved in cGnRH-II signalling, and only in the regressed stage. This observation, combined with the relative insensitivity of caffeine-stimulated hormone release to Ry, strongly suggests that RyR are found on a distinct Ca^{2+} store. Interestingly, preliminary results indicate that a competitive inhibitor of cADPr, the presumed second messenger activating RyR in many systems (reviewed in Chapter 1; Berridge, 1997), abolishes sGnRH-stimulated GH release from cell prepared from sexually regressed fish (JD

Johnson and JP Chang, unpublished; cGnRH-II has not been tested). Similarly, IP_3/xestospongin C-sensitive Ca^{2+} stores were implicated in sGnRH signalling in the same stage. This multiplicity of Ca^{2+}-dependent signalling pathways is reminiscent of cholecystokinin signalling in pancreatic acinar cells (Cancela et al., 1999). Further work is needed to determine the identity of the intracellular Ca^{2+}-release channel(s) that mediates the GH-releasing actions of both GnRHs in mature fish. As was the case in gonadotropes, several lines of evidence indicated that Tg-sensitive Ca^{2+} stores were distinct from the aforementioned intracellular Ca^{2+} pools. The distinctions between the various Ca^{2+} stores were highlighted by their selective interactions with mitochondrial Ca^{2+} handling.

Although these interpretations were based on the known pharmacology of multiple Ca^{2+} stores in cell models where it has been investigated (i.e. independence of Ry and IP_3 Ca^{2+} pools), it should be stressed that some of the 'Ca^{2+} stores' discussed in this thesis are distinguished from the others based solely on their relationship to hormone release or agonist signalling. Furthermore, except where pharmacology and/or unique inferred properties of Ca^{2+} uptake/release permit speculation, we have no direct data regarding the exact sub-cellular location of these Ca^{2+} stores. Aside from the ER, several sub-cellular locations should be considered, including the Golgi and secretory granules (as discussed in Chapter 1). Nonetheless, with the evidence reported in this thesis, goldfish gonadotropes and somatotropes are two of only a handful of cell types where more than two non-mitochondrial Ca^{2+} stores have been considered. The suggestion that goldfish pituitary cells possess more than three physiologically relevant Ca^{2+} stores is unique among pituitary cell models, to our knowledge. It is certainly curious that the complement of intracellular Ca^{2+} stores revealed in the present studies is different between goldfish gonadotropes and somatotropes. Although the reasons for the apparent cell type-specificity of certain Ca^{2+} stores are unknown, these differences represent potential specific targets for endogenous (and exogenous) regulators of gonadotrope and somatotrope function.

Although the details of GnRH signalling are clearly different between mammalian and goldfish pituitary cells, I predict that a similar level of complexity with regard to the multiplicity of agonist-sensitive Ca^{2+} stores may also eventually be uncovered in both mammalian gonadotropes and somatotropes. Unfortunately, no systematic study of this hypothesis has been reported. Moreover, studies monitoring hormone release (or other physiological endpoints) in response to modulators of multiple distinct intracellular Ca^{2+} stores are generally lacking.

Computational, biophysical and evolutionary advantages of multiple Ca^{2+} stores

What are the advantages of multiple intracellular Ca^{2+} stores? One major advantage is computational. In order to understand these advantages, one must first consider the challenges for information transduction in the hypothalamic-pituitary circuit. Both gonadotropes and somatotropes are regulated by an array of stimulatory and inhibitor hypothalamic regulators, as well as many endocrine, autocrine and paracrine factors (Chang et al., 2000; Chapter 2). As is the case in other cells, these signals likely control a vast number of cellular functions; some

of these functions would be controlled in a agonist-specific manner (as discussed in this thesis). Moreover, as are all biological signals, each of these physiological 'outputs' would be extremely complex. For example, pituitary hormone release responses, themselves, take on highly ordered and variable temporal profiles. Thus, the computational challenges on a single agonist-sensitive Ca^{2+} store would be enormous. If cells had only a single agonist-sensitive Ca^{2+} store, an idea that is still accepted by many, an information transduction bottleneck would undoubtedly occur in the signalling cascades controlling cellular functions such as exocytosis, where Ca^{2+} is thought to trigger the final step. In contrast, multiple Ca^{2+} stores which could act independently or in concert as required, could conceivably transmit a large number of independent signals simultaneously. The 'processing power' would be limited by several factors, including the ability to segregate different signals in space and time (which would depend partly on the signal-to-noise ratio in each pathway) and the number of functionally independent Ca^{2+} stores. I would argue that the signal transduction challenges encountered by a single cell are identical, in many respects, to those faced by the brain or artificial neural networks (Koch and Laurent, 1999). Thus, the paradigms of compartmentalization, parallel processing and signal complexity are directly applicable, at least conceptually to the study of single cells.

Ca^{2+} signals with sufficient spatial and temporal complexity to differentially regulate multiple cellular functions can be generated by plasma membrane ion channels alone or in combination with each other (e.g. Bading et al., 1993; Dolmetsch et al., 1998; see also Chapter 1). However, with respect to generating Ca^{2+} signal complexity, the involvement of multiple Ca^{2+} stores offers several complementary advantages over Ca^{2+} signalling systems employing only multiple extracellular Ca^{2+} influx pathways. Firstly, Ca^{2+} stores control the ion content on both sides of the membrane. Secondly, Ca^{2+} stores can send signals through the cytosol or through organelle lumens. It is likely that complex mechanisms of extracellular Ca^{2+} influx co-operate with complex systems of multiple intracellular Ca^{2+} stores (i.e. the "neuron within the neuron" concept; Berridge, 1998) to generate spatially and temporally complex Ca^{2+} signals and are sufficient to avoid a computational bottleneck.

This signalling bottleneck would also be fatal on an evolutionary timescale. As Conrad (1990) and Koch and Laurent (1999) have noted, biological structures which contain multiple components, with redundancy and weak interactions between them, are a prerequisite for optimal rates of evolution. This is because evolutionary 'tinkering' with a component that is solely responsible for an essential task is not likely to be successful. Clearly intracellular Ca^{2+} homeostasis is such a component mediating an essential task (Clapham, 1995; Berridge et al., 1998). Therefore, cell types with a single agonist-sensitive Ca^{2+} store would have less inherent evolvability. Thus, evolution may have favoured the development of complexity in Ca^{2+} signalling by creating multiple intracellular Ca^{2+} stores.

Many investigators have chosen to look for simplicity, when investigating and modeling Ca^{2+}-dependent signal transduction involving intracellular Ca^{2+}

stores. However, this may not be a valid approach for the study of signalling systems in which complexity is necessary.

Multiple intracellular Ca^{2+} stores as targets for neuroendocrine regulation

Multiple intracellular Ca^{2+} stores may be a physiologically relevant target for neuroendocrine modulation of pituitary cell function at many levels. First, as discussed above, the ability of specific extracellular signals to activate 'their own' suite of Ca^{2+} stores probably facilitates the simultaneous control of multiple cellular functions *in vivo*. This thesis presents evidence of agonist-specific Ca^{2+} stores, even when the agonists are as closely related as sGnRH and cGnRH-II. However, these *in vitro* experiments, where treatments are applied one or two at a time, are obviously a gross over simplification of the native environment when cells must deal with hundreds (at least) of extracellular signals simultaneously.

A second possible target for neuroendocrine regulation is the filling state of the intracellular Ca^{2+} stores. This may be the mechanisms by which dopamine, the major known neuroendocrine inhibitor of goldfish gonadotropes, blocks GTH-II release. Previous studies have shown that dopamine inhibits VGCC (Van Goor et al., 1998). However, acute GnRH-stimulated Ca^{2+} signals and GTH-II release are independent of VGCC (Johnson et al., 2000; Chapter 5), although Ca^{2+} entry is required to refill the agonist-stores. These findings suggest that dopamine may act by modulating the availability of intracellular Ca^{2+}. It would be interesting to vary the pretreatment time and measure the relative effectiveness of dopamine or to measure $[Ca^{2+}]_L$ in the presence of dopamine. Whether dopamine could selectively modulate some intracellular Ca^{2+} stores, but not others, also deserves experimental attention.

A third possible way by which neurohormones could target the Ca^{2+} signalling systems of gonadotropes and somatotropes is by modulating their expression or coupling to specific cellular functions (physically or biochemically). In goldfish, many of the known neuroendocrine regulators are known to display seasonal variations in their effectiveness. Although the regulation of binding sites may be important, the data presented in this thesis suggest that the properties of agonist-sensitive Ca^{2+} signalling systems may also change throughout the reproductive cycle. It is interesting to speculate, for example, that gonadal steroids may play a role in controlling the expression of RyR, as has been indicated in the rat (Sundaresan et al., 1997). Furthermore, it is possible that other hormonal inputs could inhibit or 'prime' goldfish gonadotropes by altering the properties of intracellular Ca^{2+} stores (i.e. receptor or pump expression/localization) or how Ca^{2+} stores are coupled to effectors. Such actions could produce long-term and robust changes in the dynamics of intracellular Ca^{2+} signalling in a specific cell type. For example, Ca^{2+}-release channels, such as IP_3R and RyR, as well as SERCA and PMCA, can be regulated by phosphorylation (see Chapter 1).

Differential coupling of multiple signalling pathways to secretion

We have also explored the differential coupling of Ca^{2+} stores to Ca^{2+}-dependent cellular functions, particularly exocytosis. One of the main findings is that Ca^{2+} released from Tg-sensitive Ca^{2+} stores is insufficient to stimulate acute

hormone release in either cell type. It is possible that this is a widespread phenomenon. As discussed in Chapters 10 and 12, there are currently not enough examples of Tg treatments stimulating hormone release at reasonable doses to counter such a proposal. In contrast, Ca^{2+} signals evoked by inhibition of the mitochondrial Ca^{2+} uniporter seemed to be highly coupled to secretion. These findings are further evidence of the importance of the spatial and temporal features of Ca^{2+} signals in the control of specific cellular functions (discussed more below).

Actually, there are several treatments that would be expected to increase $[Ca^{2+}]_c$ which do not stimulate hormone secretion. We have recently shown that placing cells in media containing 20 mM Ca^{2+} reduces GTH-II release (Johnson et al., 2000). In addition, it appears that treatment with 30 mM KCl can decrease GTH-II release in perifusion studies during some times of the year (Jobin, 1993; JP Chang, personal communication). We have also recently shown that 5 mM TEA reduces basal GTH-II release (CJH Wong, P Kwong, JD Johnson, Yunker WK, Chang JP, unpublished). Although they all appear to be spurious results when considered alone, the fact that three different treatments which would be expected to lead to increased Ca^{2+} influx through VGCC cause a paradoxical decrease in hormone release requires further consideration. Conversely, many treatments which would limit Ca^{2+} entry through VGCC, such as Ca^{2+}-free conditions, Cd^{2+}, nifedipine and verapamil (Johnson et al., 2000; Chapter 5; Chang et al., 1995), evoke an increase (of variable magnitude) in GTH-II release. Perhaps under conditions when intracellular Ca^{2+} homeostasis is perturbed, Ca^{2+} influx through VGCC is negatively coupled to hormone release. Exactly how VGCC are coupled to GTH-II release requires further experimentation. It is possible that Ca^{2+} microdomains, present around active VGCC, may have inhibitory effects on plasma membrane excitability, or control of Ca^{2+}-dependent enzymes which are inhibitory to exocytosis. It also interesting to note that SOC is strongly inhibited by Ca^{2+} (Barritt, 1999). Whatever the case may be, I have proposed that gonadotropes possess an exquisitely sensitive 'calcistat' than may transduce these signals.

Ca^{2+} signal coding in goldfish pituitary cells

The dissociation of Ca^{2+} signal amplitude from hormone release is also evidence of the complexity of Ca^{2+} signal coding in both cell types. The findings reported in this thesis suggests that the amplitude of global Ca^{2+} signals is not the major determinant of their effectiveness at evoking hormone release. Instead I have proposed that the rate of rise of Ca^{2+} signals may be important in the control of GTH-II release. In addition to Tg, dopamine and TEA caused slow-rising Ca^{2+} signals but inhibited GTH-II secretion. The molecular mechanism of this correlation remains unresolved. Future studies should continue to pursue the possibility that a phosphatase, preferentially activated by slow-rising Ca^{2+} signals, is responsible for modulating GTH-II release. Although the relationship between the rate of rise of Ca^{2+} signals and whether a compound is an effective secretagogue holds for gonadotropes so far, the rate of rise seems less important in somatotropes where the rate or rise of CCCP- and RR-stimulated Ca^{2+} signals

(effective secretagogues) is not significantly different from those evoked by Tg. Also, forskolin, a potent GH secretagogue, generates slow-rising Ca^{2+} signals in somatotropes (WK Yunker and JP Chang, personal communication). Interestingly, somatostatin, the major inhibitor of basal and stimulated GH release, modulates the rate of rise of some agonist-evoked Ca^{2+} signals (WK Yunker and JP Chang, unpublished). Clearly, additional determinants are involved in the selective coupling of some Ca^{2+} signals to hormone release. These additional components may be cell type- and treatment-specific.

The possibility that a population of gonadotropes and somatotropes utilizes Ca^{2+} signal frequency coding to control exocytosis or other cell functions remains to be investigated, but such investigations would require the use of more powerful tools to measure $[Ca^{2+}]_c$ and exocytosis. Moreover, although frequency coding does not appear to be prominent at the 'whole cell' level, it is quite possible that frequency coding is used at a sub-cellular level to provide added richness to localized Ca^{2+} signals.

Future directions

The present study was deliberately broad in scope as it was designed to establish the complexity of intracellular Ca^{2+} store-dependent signalling in gonadotropes and somatotropes. As a result, many unanswered questions remain.

Specifically, it would be valuable to measure Ca^{2+} levels upon application of the various treatments used in this thesis, both alone and in combination with GnRH. It will also be important to determine whether GTH-II and/or GH release by other agonists, such as PACAP, dopamine D1 and nitric oxide, is mediated by distinct Ca^{2+} stores. This information will be important, as it will allow an estimation of the ubiquity of agonist-specific Ca^{2+} stores within a single cell type. Moreover, it would be interesting to know which Ca^{2+} stores, if any, control the basal and agonist-evoked release of GTH-I from gonadotropes. In mammalian gonadotropes, the two GTHs are differentially sorted and secreted during some parts of the reproductive cycle. However, such studies in goldfish await the development of a specific RIA for GTH-I.

One of the major unaddressed areas concerns the second messengers which mobilize GnRH-sensitive Ca^{2+} stores in somatotropes. NAADP should be considered a primary candidate based on the known pharmacological sensitivities of GnRH-stimulated GH release (TMB-8, classical VGCC blockers; see Chapters 1, 5, 7). Similarly, NAADP may also play a role in signal transduction in gonadotropes. Further study of the role of cADPr in basal and agonist-evoked hormone release in both cell types is also warranted. Future work should attempt to identify the ultrastructural correlates to the agonist- and function-specific Ca^{2+} stores which have been characterized pharmacologically in this thesis. This information void represents a large obstacle on the road towards a holistic understanding of the relationship between Ca^{2+} signalling and the secretory pathway in pituitary cells.

Another interesting area of future inquiry concerns the differential modulation of gonadotrope function by the three structurally distinct SERCA inhibitors tested herein. Although preliminary trials indicated that BHQ can evoke

Ca^{2+} signals, the effect of CPA on $[Ca^{2+}]_c$ has not been examined. Such experiments may provide clues to the mechanisms underlying the selective modulation of GnRH signalling, basal hormone release and long-term hormone release, storage and production in gonadotropes. Characterization of the SERCA isoforms in gonadotropes may also be worthwhile.

At present we are limited by the existing pharmacological and molecular methods for distinguishing multiple Ca^{2+} stores. One of the main areas of limitation is the spatial and temporal resolution of our measurements. Many of the observed paradoxical relationships between global $[Ca^{2+}]_c$ elevations and hormone release can best be explained if the functional consequences of Ca^{2+} signals are decided at the level of the Ca^{2+} microdomains. Testing the hypotheses generated in this thesis will require more elaborate investigations that compare the details of Ca^{2+} signals, obtained with high spatial and temporal resolution, in response to multiple agonists, with results from simultaneous high-resolution measurements of multiple Ca^{2+}-dependent cellular functions. Although they have not been used in combination, the tools exist to simultaneously measure exocytosis, secretory pathway function, gene expression, cellular metabolism and the translocation of Ca^{2+} effector proteins with high temporal resolution. In addition, there is a requirement for methods that manipulate $[Ca^{2+}]$ in a more delicate and spatially restrictive manner than is currently possible with flash photolysis of 'caged' Ca^{2+}. Another important avenue of investigation will be to use computer-assisted modeling. Due to the complexity of multiple, simultaneously-interacting Ca^{2+} signalling cascades, mathematical modeling approaches may be advantageous, and possibly indispensable, in assembling these findings and generating testable hypotheses.

Significance

The primary goal of this thesis was to established the functional complexity of intracellular Ca^{2+} signals in goldfish gonadotropes and somatotropes. This was accomplished. In addition to advancing the understanding of the control of basal and agonist-stimulated hormone release in two neuroendocrine cell types, this thesis offers new evidence of complexity of Ca^{2+} signalling systems.

This work makes a significant contribution to the understanding of Ca^{2+}-dependent signal transduction, as well as illustrating important general principles of the coding and transduction of information in biological systems.

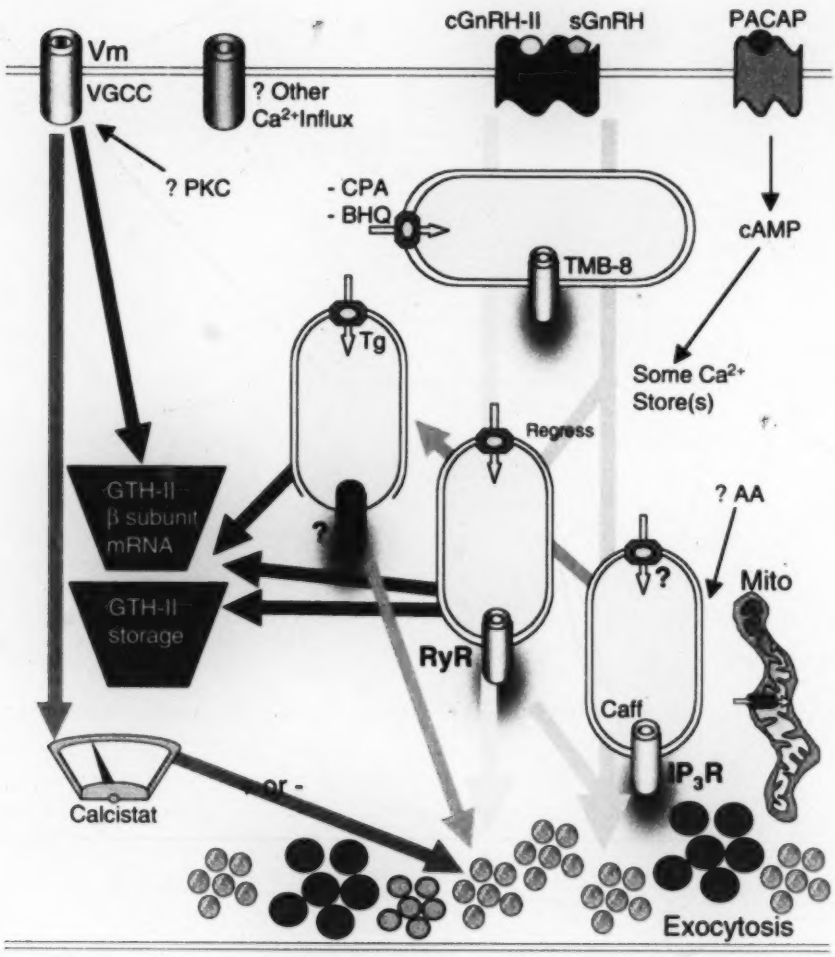

Figure 13.1. A speculative working model of the roles of multiple intracellular Ca^{2+} stores in goldfish gonadotropes. Ca^{2+} stores involved in the control of GTH-II release evoked by cGnRH-II (*yellow arrow*), sGnRH (*salmon arrow*) and PACAP (*small arrows*). Tg-sensitive Ca^{2+} store negatively regulated acute basal GTH-II release (*pale fushia arrow*), an effect which can be modulated by prior activation of a sGnRH/caffeine-sensitive Ca^{2+} store (*blue arrow*). Ca^{2+} entry through VGCC is linked to homeostatic responses (calcistat) and may be positively or negatively coupled to hormone release. Positive (*green arrows*) and negative (*red arrows*) regulation of GTH-II mRNA levels and storage are also shown.

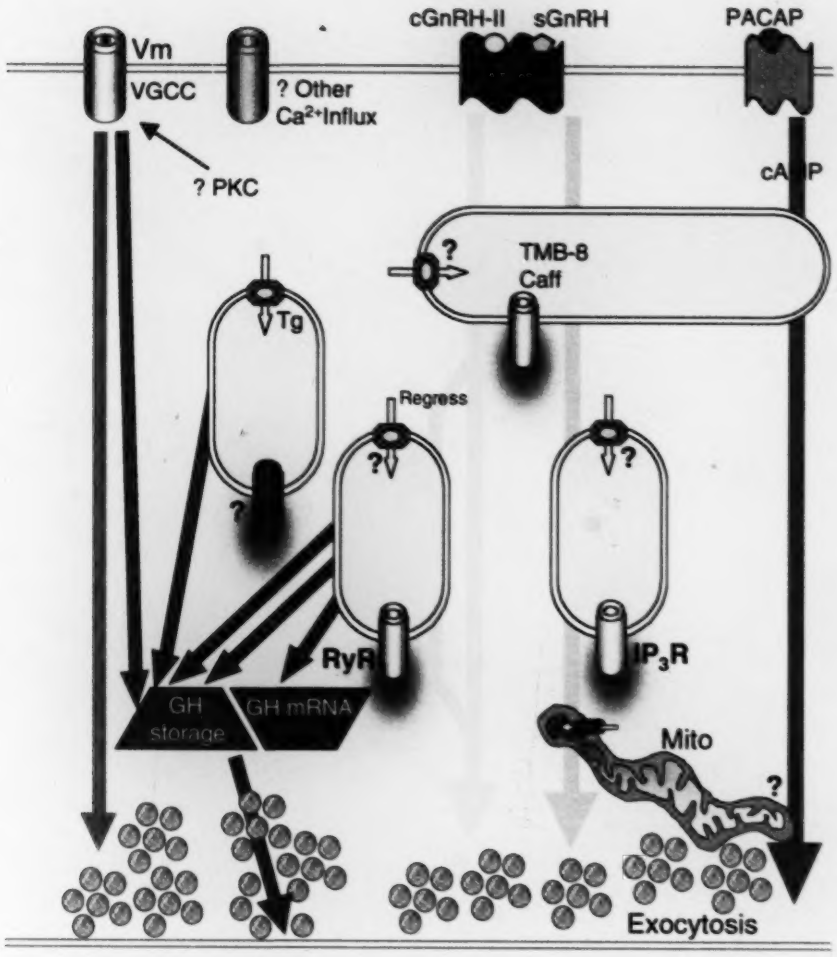

Figure 13.2. A speculative working model of the roles of multiple intracellular Ca^{2+} stores in goldfish somatotropes. Ca^{2+} stores involved in the control of GH release evoked by cGnRH-II (*yellow arrow*), sGnRH (*salmon arrow*) and PACAP (*turquoise arrow*). Mitochondrial Ca^{2+} uptake selectively interacts with sGnRH signalling. Positive (*green arrows*) and negative (*red arrows*) regulation of GH mRNA levels and storage is also shown.

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